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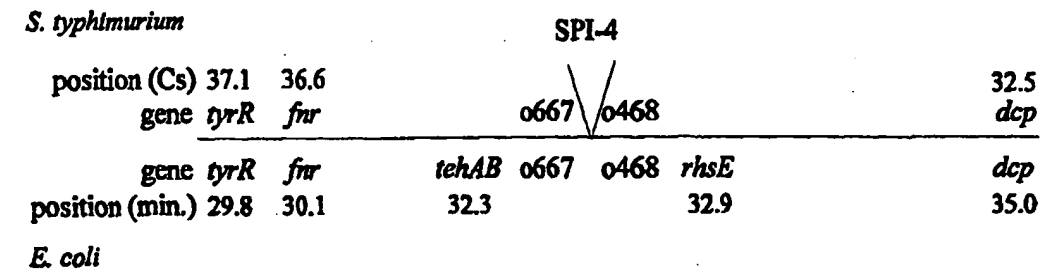

(57) Abstract

The present invention is related to a vaccine for inducing an immune response to a *Salmonella* strain in an animal, including a human, characterised in that it comprises a pharmaceutically acceptable carrier and a genetically modified *Salmonella* strain which is in an amount effective to produce an immune response in said animal, including human, and comprises a modification in its wild type DNA sequence SEQ ID NO 09, any of the DNA sequences from the same operon as a wild type DNA sequence selected from the group consisting of SEQ ID NO 01, SEQ ID NO 14, SEQ ID NO 15 and SEQ ID NO 16, and/or any regulatory sequences of any of the said DNA sequences.

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LIVE ATTENUATED SALMONELLA VACCINE

Field of the invention

The present invention relates to a pharmaceutical composition, such as a vaccine, for administration to animals, including humans, said pharmaceutical composition being able to produce an immune response against infection induced by *Salmonella* strains and/or other pathogens.

It further relates to the preparation process and to the use of said pharmaceutical composition.

State of the art

Salmonella is an important pathogen of both humans and livestock. In recent years, a steady increase has been noted of the incidence of human nontyphoidal salmonellosis, reflecting changes in animal husbandry, the mechanisation of food processing (particularly of eggs) and the mass distribution of food (Falkow S. and Mekalanos J. : The enteric Bacilli and Vibrios. In: *Microbiology*, edited by Davis, B.D., Dulbecco, R., Eisen, H.N. and Ginsberg, H.S. Philadelphia: Lippincott Co., 1990, p. 561-587). In particular, the number of human infections due to *Salmonella enteritidis* contamination of eggs and poultry meat has increased dramatically. Data of the National Reference Laboratory show that these constituted about one half of the total number of human salmonelloses in Belgium in 1996. The problem is exacerbated by the fact that the infected chickens often show no clear symptoms, while the

germ can cause a serious and potentially lethal disease in humans.

The ubiquitous presence of *Salmonella* in nature complicates the control of the disease just by detection and eradication of infected animals. Therefore vaccination of farm animals is often considered as the most effective way to prevent zoonoses caused by *Salmonella*. Different strategies were tested for the production of *Salmonella* vaccines. Inactivated cells are often not effective as vaccines. This can be explained by the fact that numerous virulence genes are tightly regulated and therefore not expressed under *in vitro* culture conditions. A more promising alternative is the use of living *Salmonella* cells, with a mutation in a gene essential for virulence, as attenuated living vaccines. Such vaccines often simultaneously elicit effective humoral, local and cellular immunity. They have the additional advantage that an oral administration is possible. This avoids the labour of injecting individual animals and is an important advantage in poultry production.

The use of several types of *Salmonella* mutants as potential live attenuated vaccines has been described, including among others:

- auxotrophic mutants, such as the *aro* mutants disclosed in patent US-5,643,771;
- mutants deficient in the production of adenylate cyclase and the cyclic AMP receptor protein, as disclosed in patent US-5,389,368
- mutants with an altered expression of outer membrane proteins, as disclosed in patent US-5,527,529
- reverse mutants of streptomycin dependant mutants, as disclosed in patent US-4,350,684
- mutants in which the regulation of gene expression is

altered by a mutation in the *phoP/phoQ* regulatory system, such as those disclosed in patents US-5,424,065 and US-5,674,736

- strains carrying one or more unidentified mutations, such as these obtained by *in vitro* passage through phagocytic cells (as disclosed in the US Patent US-5,436,001) or after mutagenesis (as disclosed in patent US-3,856,935).

The use of these strains as vaccines was often hampered by problems such as insufficient immunogenicity or excessive residual virulence.

In a number of vaccine strains, the molecular basis of the attenuating mutation is not known. For example in the US Patent US-5,436,001, a live avirulent *Salmonella choleraesuis* vaccine is disclosed. The vaccine is obtained by passing the wild-type bacteria through phagocytic cells such as macrophages or polymorphonuclear leukocytes, a sufficient number of times until the bacteria become avirulent to the animal host. However, said vaccine is very limited in its use, since the complete procedure has to be repeated with every new strain for which a vaccine is required. Moreover, since the exact nature (genotypic and/or phenotypic) of the strain modification is not explicitly known, it is not certain that the obtained strain will remain avirulent, and that its modification is transferable to other strains. There is also no routine test allowing the distinction of the vaccine strain from related virulent salmonellae.

Aims of the invention

A first aim of the invention is to provide a pharmaceutical composition such as a vaccine able to produce an immune response against a *Salmonella* strain in animals, including humans, and which does not present the drawbacks of the state of the art.

A second aim of the invention is to provide a pharmaceutical composition such as a vaccine able to produce an immune response against pathogenic agents other than *Salmonella*, infecting animals including humans, and which does not present the drawbacks of the state of the art.

Another aim of the invention is to identify sequences, involved in virulence, in *Salmonella* strains and to provide a new preparation method of an avirulent *Salmonella* strain.

Summary of the invention

The invention refers to a vaccine for inducing an immune response to a *Salmonella* strain in an animal, including a human, said vaccine comprising a pharmaceutically acceptable carrier and one or more genetically modified *Salmonella* strain(s) in an amount effective to produce said immune response (humoral, local and/or cellular immune response) and wherein said genetically modified *Salmonella* strain comprises a modification in its wild type DNA sequence SEQ ID NO 09, its complementary strand, or in a homologous sequence, said modification being preferably in SEQ ID NO 1, SEQ ID NO 14, SEQ ID NO 15 or SEQ ID NO 16.

Indeed, the Inventors have discovered that a *Salmonella* strain comprising a modification in its wild type DNA sequence SEQ ID NO 01 and/or its complementary strand becomes avirulent.

Said isolated and/or purified wild type DNA sequence SEQ ID NO 01 is identified in the enclosed sequence listing, and the genetic modification of said isolated and/or purified wild type DNA sequence SEQ ID NO 01 is preferably an insertion, a deletion and/or a substitution of at least one nucleotide in said DNA sequence.

The Inventors have discovered unexpectedly that it is possible to reduce the "virulence" of a *Salmonella* strain by a genetic modification of said wild type DNA sequence. This sequence directly or indirectly promotes the virulence of *Salmonella* strains.

The "virulence" of the pathogen (*Salmonella* strain according to the invention) means the induction in an animal (including human) of infection and symptoms (salmonellosis) due to *Salmonella* contamination.

It is clear that genetic modifications in any DNA sequence belonging to the same operon than the DNA sequence as in SEQ ID NO 01, SEQ ID NO 14, SEQ ID NO 15 or SEQ ID NO 16, including its complementary strand and/or genetic modifications in any regulatory sequence of any of the said DNA sequences, may also result in a reduction in virulence of *Salmonella* strains as described above.

Further, the vaccine according to the invention can comprise supplementary genetic modification in other gene regions than the operons described hereabove. Preferably, said supplementary genetic modification is a mutation in the spiC, aro, pur, dap, pab, sipC, phoP, phoQ and/or pagC gene regions.

In the vaccine according to the invention, the pharmaceutically acceptable carrier can be any compatible non-toxic substance suitable for administering the composition (vaccine) according to the invention.

The pharmaceutically acceptable carriers

according to the invention suitable for oral administration are the ones well known by the person skilled in the art, such as tablets, coated or non-coated pills, capsules, solutions or syrups. Other adequate pharmaceutical carriers or vehicles may vary according to the mode of administration (intravenous, intramuscular, parenteral, etc.).

The vaccine according to the invention may comprise also adjuvants well known by the person skilled in the art which may increase or regulate the humoral, local and/or cellular response of the immune system against *Salmonella* strains, other pathogenic agents or other epitopes. The vaccine according to the invention is prepared by the methods generally applied by the person skilled in the art for the preparation of a vaccine wherein the percentage of the active compound/pharmaceutically acceptable carrier can vary within very large ranges, only limited by the tolerance and the level of acquaintance of the patient to the vaccine. The limits are particularly determined by the frequency of administration.

Advantageously, the genetically modified *Salmonella* strain in the vaccine according to the invention may also comprise an isolated (and preferably purified) nucleotide sequence encoding a *Salmonella*-foreign antigen and said genetically modified *Salmonella* strain is present in the vaccine in an amount effective to induce an immune response to said *Salmonella*-foreign antigen.

The isolated (and preferably purified) nucleotide sequences encoding *Salmonella*-foreign antigens are the ones well known by the person skilled in the art and described in the scientific literature and known to induce an immune response against pathogenic agents such as bacteria, viruses or eukaryotic pathogenic agents which may induce infectious diseases in animals, including humans, or

against other epitopes or epitope-bearing entities such as tumor antigens or portions thereof or a combination thereof, hormones, allergens, toxins, etc.

Preferably, the genetically modified *Salmonella* strain according to the invention is selected from the group consisting of the following *Salmonella* : *Salmonella enteritidis* (preferably *Salmonella enteritidis* EZ1263 having the deposit number LMGP-18112), *Salmonella typhimurium*, *Salmonella choleraesuis*, *Salmonella dublin*, *Salmonella paratyphi*, *Salmonella typhi*, *Salmonella hadar*, *Salmonella infantis*, *Salmonella montevideo* and *Salmonella senftenberg*.

Another aspect of the present invention is related to a (preferably virulent) isolated or synthetic nucleotide sequence having at least 55 % homology with the wild type DNA sequence SEQ ID NO 09, between positions 163 and 3580, or its complementary strand, or in a homologous sequence.

Another aspect of the present invention is related to a (preferably virulent) isolated or synthetic nucleotide sequence having at least 40 % homology with the wild type DNA sequence SEQ ID NO 01 or its complementary strand, or in a homologous sequence.

Another aspect of the present invention is related to a (preferably virulent) isolated or synthetic amino acid sequence having at least 30% homology with the wild type amino sequence SEQ ID NO 02.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring).

The variant of the SEQ ID NO 01 may be a naturally occurring allelic variant of SEQ ID NO 01 or a non-naturally occurring variant of SEQ ID NO 01.

As known in the art, an allelic variant is an

alternate form of a sequence which may have a substitution, deletion or addition of one or more nucleotides and/or amino acids which preferably does not substantially alter the function of the encoded polypeptide.

A "virulent" genetic sequence is a nucleotide or amino acid sequence that is important for the infectious ability of a pathogen.

Said sequences may present an industrial application in the field of diagnostic (identification of various virulent increasing virulent salmonella strains) or for development of an avirulent vaccine comprising said sequences.

A further aspect of the present invention concerns a preparation method of an avirulent *Salmonella* strain, comprising the steps of:

- identifying a "virulent" nucleotide sequence in the genome of a *Salmonella* strain by any method based on the use of nucleotide sequence SEQ ID NO 09 or the complementary strand thereof, such as hybridisation or amplification by the polymerase chain reaction with a probe or primers having at least 12 nucleotides and which shows at least 10 identical nucleotides with a corresponding portion of SEQ ID NO 09 or its complementary strand or which shows more than 50% homology with a corresponding portion of SEQ ID NO 09 or its complementary strand.
- inducing a modification in said "virulent" nucleotide sequence in order to obtain an avirulent or less virulent nucleotide sequence, and
- recovering an obtained avirulent *Salmonella* strain having said modification in its "virulent" sequence.

In a preferred embodiment of the present invention, said hybridization is obtained under standard stringent hybridization conditions or which would hybridize

for the redundancy of the genetic code.

Exemplary stringent hybridization conditions are as follows: hybridization at 42°C in 50% formamide, 5X SSC, 20 mM sodium phosphate, pH 6.8 washing in 0.2X SSC at 55°C. It is understood by those skilled in the art that variation in these conditions occurs based on the length and GC nucleotide content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining exact hybridization conditions. *See Sambrook et al.*, §§ 9.47-9.51 *in* Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

The present invention is also related to a method for inducing an immune response to a *Salmonella* strain in an animal, including a human, comprising administering a pharmaceutical composition preferably comprising a pharmaceutically acceptable carrier and a live, genetically modified *Salmonella* strain to said animal, wherein said genetically modified *Salmonella* strain is in an amount effective to produce an immune response and wherein said genetically modified *Salmonella* strain comprises a modification in its wild type DNA sequence SEQ ID NO 09 and/or its complementary strand. Said genetically modified *Salmonella* strain is preferably administered in a pharmaceutically acceptable carrier.

In the method according to the invention, the modification of the "virulent" sequence is preferably obtained by an insertion, a deletion and/or a substitution of at least one nucleotide in said nucleotide sequence. Said insertion, deletion or substitution is preferably obtained by homologous recombination with an engineered nucleotide sequence, comprising said insertion, deletion or substitution.

The present invention is also related to the

use of the pharmaceutical composition, preferably the vaccine according to the invention, for the preparation of a medicament for inducing an immune response to a *Salmonella* strain in an animal, including a human, preferably for inducing therapeutic and/or protective properties against a *Salmonella* strain and avoid salmonellosis diseases.

Advantageously, said immune response is an effective humoral, local and/or cellular immune response.

Another aspect of the present invention is a vaccine for inducing an immune response to a *Salmonella* strain in an animal, including a human, said vaccine comprising a pharmaceutically acceptable carrier and one or more genetically modified *Salmonella enteritidis* strain(s) in an amount effective to produce said immune response (humoral, local and/or cellular immune response) and wherein said genetically modified *Salmonella* enteritidis strain comprises a modification in its wild type spiC DNA sequence, its complementary strand, or in a homologous sequence.

Preferably, said modification is in SEQ ID NO 13.

In a preferred embodiment, said genetically modified *Salmonella* enteritidis is EZ870, having the deposit number LMGP-18484.

The present invention will be described in details in the following examples, in reference to the following figures which are presented as illustration of the various embodiments of the present invention without limiting its scope.

Short description of the drawings

Figure 1 represents a schematic overview of the region

of the *Salmonella* chromosome containing the transposon insertion in *S. enteritidis* EZ1263, showing the orientation and relative organisation of the genes in this chromosomal region.

Figure 2 represents a schematic overview of the comparison between the *E. coli* genetic map and the *S. typhimurium* genetic map of the region containing the transposon insertion in *S. enteritidis* EZ1263, showing the closest mapped genes in *E. coli* and *S.typhimurium*.

Figure 3 represents the result of an ELISA test, showing that antibodies directed against *Escherichia coli* F17 fimbriae are produced after infection of mice with *S. enteritidis EZ1263* producting these fimbrae.

Figure 4 represents the result of an ELISA test, showing that antibodies directed against *S. enteritidis* lipopolysaccharides are produced after infection of mice with *S. enteritidis EZ1263* producing *Escherichia coli* F17 fimbrae.

Examples

Example 1 : Construction of the attenuated S. enteritidis mutant EZ1263

The *S. enteritidis* phage type 4 strain 76Sa88 (a clinical isolate from a chicken, obtained from the Veterinary and Agrochemical Research Centre, Groeselenberg 99, B-1180 Ukkel, Belgium) was used for the isolation of attenuated transposon insertion mutants. To facilitate the selection, the spontaneous rifampicin resistant mutant 76Sa88Rif^R was first isolated by plating samples of an

overnight culture of 76Sa88 in LB medium (Miller, J.H. *Experiments in Molecular Genetics*, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1972. pp. 1-466) onto LB plates containing 100 mg/l rifampicin. Oral infection of Balb/c mice confirmed that the rifampicin resistance mutation of the strain 76Sa88Rif^R does not affect its virulence.

Mutants of 76Sa88Rif^R were isolated by insertion mutagenesis using the transposon miniTn*5lacZ2*, that generates translational fusions with the β-galactosidase gene *lacZ* (de Lorenzo V., Herrero M., Jakubzik U., Timmis K.N., J. Bacteriol. 172 (11): 6568-6572, 1990). This system allows the identification of insertion mutations in genes that show a particular regulation pattern. The transposon miniTn*5lacZ2* is harboured on the suicide plasmid pUT that is unable to replicate in *Salmonella*. Transposon miniTn*5lacZ2* insertion mutants were obtained by conjugation of *E. coli* S17-1(λ*pir*) (Simon R., Priefer U., and Pühler A., Biotechnology 1, 784-791, 1983), harbouring the pUT plasmid containing the miniTn*5lacZ2* transposon, with 76Sa88Rif^R on LB medium. The insertion mutants were selected subsequently on LB plates with 100 mg/l kanamycin (marker of miniTn*5lacZ2*) and 100 mg/l rifampicin. After colony purification, the mutants were tested for growth on LB medium with 100 mg/l carbenicillin (marker of pUT) to confirm the loss of the suicide plasmid. The carbenicillin-sensitive clones were cultured in 96-well microplates and replicated on different media simulating the conditions in the host. These media also contained the β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), that produces a blue precipitate upon hydrolysis.

In one of the insertion mutants, strain EZ1263, β-

galactosidase expression was induced by culture on the defined medium Minimal A (Miller, J.H. *Experiments in Molecular Genetics*, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1972. pp. 1-466), by culture at low pH (LB medium, buffered at pH 5.5 with 0.1 M MES (2-[N-Morpholino]ethanesulfonic acid) and by the iron chelator dipyridyl (0.2 mM). This induction pattern suggested that the corresponding gene might also be expressed *in vivo* after infection of the animal host, as is predicted for a virulence factor. Therefore the pathogenicity and immunogenicity of the strain EZ1263 were tested in mice (See following examples 3, 4, 5, 7 and 8).

A deposit has been made according to the Budapest Treaty for the micro-organism *Salmonella enteritidis* EZ1263 under deposit number LMGP-18112 at the BCCM/LMG Culture Collection, Laboratorium voor Microbiologie, Ledeganckstraat 35, B-9000 Gent (Belgium)

Example 2: Identification of the mutation causing the attenuation of S. enteritidis EZ1263

To analyse the mutated gene in EZ1263, total genomic DNA of the mutant was prepared (Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Seidman J.D., Smith J.A. and Struhl K. *Current Protocols in Molecular Biology*. Wiley Interscience, 1987), digested to completion with the restriction enzyme *Taq*I and circularised under conditions that favour intra-molecular ligation (1 µg DNA in a total reaction volume of 200 µl, using 0.1 unit of T4 DNA ligase, incubation overnight at 4 ^{o}C). This circular DNA was immediately used as template in IPCR amplification. PCR primers were designed according to the fusion fragment obtained after *Taq*I digestion, containing the first 836 bp

of the *lacZ* coding sequence. Four *lacZ*-specific primers, forming two nested pairs, were synthesised (see Table 1).

Table 1 : Synthetic oligonucleotides used as PCR primers

Primer	Sequence from 5' to 3'	Restriction site	Position	SEQ ID NO
lacZ1*	GGGAATTCAAAGCGCCATTCGCCATTCAG	*Eco*RI	1467-1446	3
lacZ2*	GGAAGCTTTATGGCAGGGTGAAACGCAGG	*Hind*III	2062-2085	4
lacZ3*	CGTCTAGACGTTTTCCCAGTCACGAC	*Xba*I	1340-1324	5
lacZ4*	GCGGATCCTTTCGGCGGTGAAATTATC	*Bam*HI	2103-2123	6
1263-1**	ACAGACGATTTTTCTCTA	-	-	7
1263-2B**	CGCCCCATTAAAGGCTAT	-	-	8

* *lacZ*-specific PCR primers with their incorporated restriction enzyme sites (underlined) and their position in the *E. coli lacZ* gene (ECOLAC, Accession number: J01636)

** SEQ ID 01-specific primers

The IPCR reaction mixture consisted of 0.4 µM primer lacZ1, 0.4 µM primer lacZ2, template DNA (5 µl intra-molecular ligation mixture), 200 µM of each dNTP and 0.1 unit Super*Tth* Taq DNA polymerase (H.T. Biotechnology) in 50 µl Tth buffer (H.T. Biotechnology). The reaction conditions were as follows: three initial cycles of 94 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min, followed by 35 cycles of 94 °C for 30 sec, 53 °C for 30 sec and 72 °C for 1 min. Gel-purified IPCR products (Jetsorb Gel Extraction kit, Genomed) were reamplified with nested primers lacZ3 (0.4 µM) and lacZ4 (0.4 µM) in a 50 µl reaction mixture as previously described. Twenty-five re-amplification cycles were done at 94 °C for 30 sec, 53 °C for 30 sec and 72 °C for 1 min. The IPCR and PCR

amplifications were successful, using a Koch Light N.B.S. TC-1 thermocycler or a Perkin-Elmer GeneAmp PCR system 9600.

After re-amplification, the PCR products were directly sequenced using the Sequenase™ PCR Product Sequencing Kit (USB/Amersham). Starting with 0.2-0.5 pmol template, DNA sequencing was performed with the pUC forward primer provided in the kit, as well as with the lacZ4 primer.

The obtained PCR product has an estimated size of 650 bp. The 317 nucleotides upstream of the transposon were sequenced using the pUC forward primer and the lacZ4 primer. A search for homologous sequences in the bacterial DNA database was done with the obtained nucleotide sequences, using the FastA programme (Sequence Analysis Software Package, Genetics Computer Group, Inc.) and revealed no homologies with any of the known sequences.

To obtain the wild-type sequence corresponding to the mutated gene in EZ1263, a cosmid library of *S. enteritidis* genomic DNA was screened, using the re-amplification PCR-product as a probe specific for the mutated gene. The construction of the cosmid library and the procedure for colony hybridisation were described (Woodward M.J., Allen-Vercoe E., Redstone J.S., Epidemiol Infect 117 (1): 17-28, 1996). Fixation of the DNA on the Hybond N membrane was done by UV cross-linking. The PCR product obtained for mutant EZ1263 was used as a probe for hybridisation, after re-amplification with primers lacZ3 and lacZ4 and purification from an agarose gel (Jetsorb Gel Extraction kit, Genomed). The Ready To Go DNA labelling Beads (Pharmacia Biotech) were used to radio-label 25-30 ng of the purified PCR fragment. Unincorporated radioactive nucleotides were separated from the labelled probe with a

ProbeQuant™ G-50 Micro Column (Pharmacia Biotech). During the labelling procedure, the hybridisation membranes were prehybridised in RapidHyb (Amersham) buffer at 65 °C (30-60 min.). The labelled probe was added to the membranes and hybridisation continued for 2-3 h. at 65 °C. After hybridisation, the membranes were washed: once 20 min. in 0.3 M NaCl, 0.03 M Na_3-citrate, 0.1 % SDS at room temperature and twice 15 min. at 65 °C in 0.03 M NaCl, 3 mM Na_3-citrate, 0.1 % SDS. The signal was detected by putting an X-ray film on top of the membrane and incubating at room temperature.

Four cosmid clones showed hybridisation with the EZ1263-probe and were sorted from the cosmid library stock. Cosmid DNA was prepared using the Qiagen Plasmid Midikit as described in the manual. The obtained DNA was digested with several restriction endonucleases, separated by agarose gel electrophoresis and used for Southern blotting by standard procedures. Prehybridisation, hybridisation and labelling of the probe was carried out as described above. This hybridisation confirmed the homology of clones 3B7 and 4F9 with the EZ1263-probe. Furthermore it showed that the probe hybridised with a 1.7 kb *Pst*I fragment, a 4.5 kb *Eco*RV fragment and a 8 kb *Bam*HI fragment. For the endonucleases *Bgl*II, *Eco*RI and *Hin*dIII the size of the hybridising fragment was larger than 12 kb.

The 1.7 kb *Pst*I fragment was cloned into the *Pst*I site of the vector pUC19 using standard techniques. This resulted in the vector pGV4357. Several deletions in the insert were constructed to facilitate sequencing of the complete 1.7 kb *Pst*I fragment. Sequence analysis was carried out using the SequiTherm Cycle sequencing kit (Epicentre Technologies) or the Pharmacia ALF automatic DNA sequencer or the ThermoSequenase radio-labelled terminator

cycle sequencing kit (Amersham). The transposon insertion in mutant EZ1263 is located in an open reading frame of 771 base pairs (*orfX* = SEQ ID NO 01), encoding a prospective protein of 257 amino acids (SEQ ID NO 02).

Screening of the nucleotide sequence databases with the nucleotide sequence SEQ ID NO 01, using the programmes BLASTN (Altschul S.F., Gish W., Miller W., Myers E.W., and Lipman D.J., J. Mol. Biol. 215, 403-10, 1990) and FastA (Sequence Analysis Software Package, Genetics Computer Group, Inc.), confirmed the absence of *Salmonella* sequences with a significant homology. The most related nucleotide sequence detected by BLASTN was the *vanX* gene of transposon Tn*1546* of *Enterococcus faecalis*, encoding a D-alanyl-D-alanine dipeptidase involved in vancomycin resistance (accession number M97297). Alignment of the coding sequence of *vanX* with SEQ ID 01 (using the PCgene programme NAlign with open gap cost 50 and unit gap cost 10) resulted in 298 identical nucleotides (38.6 %). Alignment of the amino acid sequence of VanX with SEQ ID 02 (using the PCgene programme PAlign, comparison matrix: Dayhoff MDM-78 with open gap cost 200 and unit gap cost 100) resulted in 71 identical residues (27.7 %) and 38 similar residues.

Within the *Enterobacteriaceae*, the highest degree of sequence identity was found using TBLASTX (that compares a nucleic acid sequence translated in the six translation frames against a nucleic acid database translated sequence by sequence in the six translation frames) with an *E. coli* sequence (accession number AE000245) that was determined as part of the *E. coli* Genome Project. The homologous sequence encodes a putative protein, called f193, of 193 amino acids that is 41 % identical (22 gaps) to 154 residues from D-alanyl-D-alanine dipeptidase VanX. Alignment of the coding sequence of f193

with SEQ ID NO 01 (using the PCgene programme NAlign with open gap cost 50 and unit gap cost 10) resulted in 259 identical nucleotides (33.5 %). Alignment of the amino acid sequence of f193 with SEQ ID NO 02 (using the PCgene programme PAlign, comparison matrix: Dayhoff MDM-78 with open gap cost 200 and unit gap cost 100) resulted in 62 identical residues (24.2 %) and 29 similar residues.

In order to obtain more information on the DNA sequence adjacent to *orfX*, the 8 kb *Bam*HI fragment of the cosmid clone p3B7 was cloned in the *Xho*I site of the plasmid vector pBluescriptSK$^-$. For this aim, the size of the cosmid clone p3B7 was first reduced by *Bam*HI digestion followed by self-ligation. The resulting plasmid, pGV4437, contains the SuperCosI vector and the 8 kb *Bam*HI fragment. Plasmid DNA of pGV4437 was digested with *Bam*HI and the cohesive ends of the fragments were partially filled-in using dGTP and dATP. Plasmid DNA of pBluescriptSK$^-$ was digested with *Xho*I and the cohesive ends were partially filled-in using dCTP and dTTP. Ligation of the filled-in 8 kb *Bam*HI fragment in this vector resulted in the plasmid pGV4563.

Sequence analysis of the cloned 8 kb *Bam*HI fragment revealed that *orfX* lies within a region of the *Salmonella* chromosome that has no homologue in the *E. coli* chromosome. The genetic maps of *Salmonella typhimurium* and *E. coli* K12 are highly conserved but differences in the gene intervals between the two genomes have been observed and are called loops (Riley and Sanderson, 1990. in Drlica and Riley (ed.) The bacterial chromosome, American Society for Microbiology, Washington D.C. p 85-95).

The *orfX* is located at the end of the loop that has a length of 3417 bp and is located from bp 163 to 3580 in SEQ ID NO 09.

Using the BLASTN program with default

settings (Altschul *et al.*, J. Mol. Biol. 215: 403-410., 1990), to screen the GenEMBL DNA database, it was found that the loop is flanked by sequences that show homology with the *E. coli* open reading frames f76 and o468 (Accession number AE000241, Blattner *et al.*, Science 277, 1453-1462, 1997). The sequence flanking *orfX* has 71 % (218/303) identical base pairs with o468 and the sequence flanking the other boundary of the loop has 71 % (113/159) identical base pairs with f76.

In addition to *orfX*, three more putative open reading frames were identified in the loop. A schematic overview of the genes in the loop is given in figure 1.

The open reading frame *orfA* is in divergent orientation of *orfX* and located at the other end of the loop, adjacent to the f76 homologue. The *orfA* open reading frame, starting at nucleotide 1302 of SEQ ID NO 09 and ending at nucleotide 352 of SEQ ID NO 09, encodes a hypothetic protein of 316 amino acids (SEQ ID NO 10). Screening the GenEMBL DNA database, using the FastA programme (Sequence Analysis Software Package, Genetics Computer Group, Inc.) with word size 6, revealed that the open reading frame *orfA* has 51.8% identical nucleotides in a 662 bp overlap with the *sifA* gene of *Salmonella typhimurium* (Accession number U51867). The hypothetic gene product of *orfA* showed 26.7 % identical amino acids in a 307 amino acid overlap, with the *S. typhimurium sifA* gene product (Accession number U51867, Stein *et al.* Mol. Microbiol., 20: 151-164, 1996), using the TFastA programme (Sequence Analysis Software Package, Genetics Computer Group, Inc.) for comparing the amino acid sequence of the hypothetic *orfA* gene product with the GenEMBL databank. The *S. typhimurium sif*A gene is involved in the production of "*Salmonella* induced filaments" in infected epithelial cells and is required for the full virulence of this bacterium

(Stein *et al.*, Mol. Microbiol., 20: 151-164, 1996).

The sequences of *orfX* and *orfA* are in divergent orientation and are separated by a 1450 bp region, containing two putative open reading frames, *orfV* (399 bp) and *orfW* (231 bp). Open reading frame *orfV* starts at nucleotide 1839 of SEQ ID NO 09, ends at nucleotide 2237 of SEQ ID NO 09 and encodes a hypothetic protein of 133 amino acids (SEQ ID NO 11). Open reading frame *orfW* starts at nucleotide 2270 of SEQ ID NO 09, ends at nucleotide 2500 of SEQ ID NO 09 and encodes a hypothetical protein of 77 amino acids (SEQ ID NO 12). No sequences with significant homology to *orfV*, *orfW* or the encoded gene products could be found in the EMBL database with the programmes FastA, TFastA BLASTN, BLASTP or BLASTX (Sequence Analysis Software Package, Genetics Computer Group, Inc., Altschul *et al.*, J. Mol. Biol. 215: 403-410., 1990).

The sequence, comprising *orfA*, *orfV*, *orfW* and *orfX* is not homologous with the region between f76 and o468 in the *E. coli* genome. This 3417 bp *Salmonella* loop has an aberrant G+C content of 40.5 % instead of 52-54 %, wich is the average G+C content of the *Salmonella* genome (Ochman H. and Lawrence J.G., in: *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology, Neidhardt F.C. *et al.* eds, ASM press vol 2, p2627-2637, 1996) The neighbouring genes have G+C contents of 51.9 % for the f76-homologous gene and 53.9 % for the o468-homologous gene.

These data prove that this 3417 bp region is located on a new *Salmonella*-specific loop, comprising a *Salmonella* pathogenicity islet (Groisman & Ochman, Trends Microbiol. 59: 343-349, 1997).

The chromosomal location of the Salmonella-specific loop containing *orfA*, *orfV*, *orfW* and *orfX* was deduced by comparing the genetic map of *E. coli* (Berlyn *et al.*, 1996 in *Escherichia coli* and *Salmonella typhimurium*:

Cellular and Molecular Microbiology, Neidhardt F.C. *et al.*, eds, ASM press, vol 2, p1715-1902, 1996) and *S. typhimurium* (Sanderson *et al.*, 1996 in *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Microbiology, Neidhardt *et al.*, eds, ASM press, vol 2, p1903-1999, 1996). The *E. coli* genes f76 and o468, that are homologous to the *Salmonella* genes flanking the loop, are located on the *E. coli* chromosome in the region between the genes *tehAB* at 32.3 min of the *E. coli* chromosome on one side and *rhsE* at 32.9 min on the other side. For these genes no *Salmonella* homologues have been mapped. The closest mapped genes with mapped *Salmonella* homologues are the *fnr* gene located at 30.1 min. in *E. coli* and 36.6 Cs in *S. typhimurium*, and the *dcp* gene located 35.5 min. in *E. coli* and at 32.5 Cs in *S. typhimurium* (see figure 2). Thus the 3417 bp *Salmonella* specific loop, containing *orfA*, *orfV*, *orfW* and *orfX*, is located between 35.5 Cs and 36.6 Cs on the *Salmonella* chromosome.

Example 3 : <u>Induction of protective immunity against S. enteritidis after intra-peritoneal vaccination of mice with S. enteritidis EZ1263</u>

S. enteritidis EZ1263 was cultured overnight at 37 °C in LB medium, spun down and resuspended in PBS (1.5 mM KH_2PO_4, 10 mM Na_2HPO_4, 140 mM NaCl, 3 mM KCl, pH 7.2). Intraperitoneal injection of 2.1 10^2 colony forming units of these EZ1263 bacteria in 5-6 week old female Balb/c mice did not produce perceptible disease symptoms, while all control mice injected with 2.3 10^2 colony forming units of the wild type *S. enteritidis* 76Sa88 under identical conditions were killed by the infection (see Table 2).

Table 2 : Results of intra-peritoneal infection of mice with wild type *S. enteritidis* 76Sa88 or mutant EZ1263

Strain	Dose (cfu)	Number of surviving mice	Dead days after inoculation
EZ1263	$2.1\ 10^2$	3/3	/
76Sa88	$2.3\ 10^2$	0/3	9, 9, 9
Negative control	0	3/3	/

The mice that were intraperitoneally injected with strain EZ1263 were submitted to an oral challenge of wild type *S. enteritidis* 76Sa88 after 34 days. The germs were cultured overnight in LB broth at 37 °C, spun down and resuspended in milk. $2.8\ 10^8$ colony forming units (in 50µl milk) were applied using a micropipette. This corresponds with about 10^5 LD_{50} units. All of the injected mice survived the challenge infection without observable disease symptoms, while all of non-injected control mice that were orally challenged under identical conditions were killed (see Table 3).

Table 3 : Protection against oral infection with wild type *S. enteritidis* 76Sa88 after intraperitoneal (IP) infection with mutant EZ1263

Previous IP. Infection	Dose of 76Sa88 (cfu)	Number of surviving mice	Dead days after inoculation
EZ1263	$3.3\ 10^8$	3/3	/
Negative control	$3.3\ 10^8$	0/2	6, 8

These data show that intraperitoneal vaccination of Balb/c mice with strain EZ1263 induces protective immunity against *S. enteritidis* phage type 4.

Example 4 : Induction of protective immunity against S. enteritidis after oral vaccination of mice with *S. enteritidis* EZ1263

Nine female Balb/c mice, 5 to 6 weeks old, were orally infected with EZ1263 bacteria (by the method described in example 3) in three independent experiments, using a dosage of $1.4\ 10^8$, $3.4\ 10^8$ and $1.6\ 10^8$ colony forming units respectively. All of the infected animals survived without any clear disease symptoms, while all control mice infected with the wild type *S. enteritidis* 76Sa88 under identical conditions were killed by the infection (see Table 4).

Table 4 : Oral infection of mice with wild type *S. enteritidis* 76Sa88 or mutant EZ1263

Strain	Dose (cfu)	Number of surviving mice	Dead days after inoculation
EZ1263*	$3.4\ 10^8$	3/3	/
EZ1263**	$1.3\ 10^8$	3/3	/
EZ1263***	$1.6\ 10^8$	3/3	/
76Sa88*	$3.8\ 10^8$	0/3	6, 6, 7
76Sa88**	$2.3\ 10^8$	0/3	7, 8, 8
Negative control	0	5/5	/

*, **, ***: Results of three independent experiments

Six mice that were orally vaccinated with strain EZ1263 were orally challenged with wild type *S. enteritidis* 76Sa88 (by the method described in example 3) in two independent experiments. The dose administered was $1.6\ 10^8$ and $3.3\ 10^8$ colony forming units respectively. The challenge was carried out 15 respectively 33 days after the oral vaccination with EZ1263. The vaccinated mice survived the challenge infection without showing clear disease symptoms, while all non-vaccinated control mice that were orally challenged under identical conditions were killed (see Table 5).

Table 5 : Protection against oral infection with wild type *S. enteritidis* 76Sa88 after oral infection with mutant EZ1263

Previous oral infection	Dose of 76Sa88 (cfu)	Number of surviving mice	Dead days after inoculation
EZ1263*	$1.6\ 10^8$	3/3	/
none*	$1.6\ 10^8$	0/2	8, 9
EZ1263**	$3.3\ 10^8$	3/3	/
none**	$3.3\ 10^8$	0/3	9, 10, 11
Negative control	0	1/1	/

*, **: Results of two independent experiments

These data show that oral vaccination of Balb/c mice with strain EZ1263 induces protective immunity against *S. enteritidis* phage type 4.

Example 5 : Induction of humoral immunity after oral vaccination of chicks with S. enteritidis EZ1263

Twelve one day old SPF (specific pathogen free) chicks were orally infected with 10^9 colony forming units of EZ1263 bacteria (cultured for 20 hours in Brain Hearth Infusion broth at 37 °C). Twelve one day old SPF chicks were simultaneously orally infected with 10^9 colony forming units of the wild type *S. enteritidis* 76Sa88 under identical conditions. Eleven of the 12 chicks infected with EZ1263 survived the infection with minimal disease symptoms and minimal growth retardation. Only 2 in 12 chicks infected with 10^9 colony forming units of the *S. enteritidis* 76Sa88 survived the infection. These showed severe disease symptoms and growth retardation (see Table 6).

Table 6 : Death and symptoms after oral infection of one day old chicks with wild type *S. enteritidis* 76Sa88 or mutant EZ1263

Strain	Number of surviving chicks	General depression	Diarrhoea	Growth retardation	Average weight (g)
EZ1263	11/12	0-1	2	0-1	258 ±56
76Sa88	2/12	4	5	3	196 ±51

The clinical symptoms are represented with a score: 0: no symptoms, 1 to 5: light to very clear symptoms. The average weight of the chicks still alive 28 days after infection is given with the standard deviation.

Serum samples of the 11 chicks vaccinated with EZ1263 were taken 4 weeks after infection to test the presence of anti-*Salmonella* antibodies by ELISA essentially as described (Desmidt M., Ducatelle R., Haesebrouck F., de Groot P.A., Verlinden M., Wijffels R., Hinton M., Bale J.A., Allen V.M., Vet. Rec. 138 (10): 223-226, 1996). Microtitre plates (96 wells) were coated with complete *S. enteritidis* (20 hours culture in Brain Hearth Infusion broth at 37 °C, washed in PBS and killed with 99.5 % acetone) or with *S. enteritidis* Lipopolysaccharide (LPS), prepared as described by Westphal O. and Jann K. (in Methods in carbohydrate chemistry, Whistler RL and Wolfrom ML (eds) Academic Press, London p 83-99, 1965). The coating was performed using an antigen solution (10 µg/ml, 150 µl/well) in carbonate/bicarbonate buffer at pH 9.6 for 24 hours at 4 °C. The plates were rinsed once with rinsing buffer (0.05 % Tween 20 in PBS) and blotted on a paper towel. The chick sera were diluted 1:200 in rinsing buffer with 2.2% skimmed milk powder for the ELISA

with the LPS antigen and 1:500 for the ELISA with the complete germ. These diluted sera were incubated on the coated plates for 2 hours at 37 °C. After five rinses, the plates were incubated for 30 min. with rabbit anti chicken immunoglobulin conjugated with horseradish peroxidase (diluted 1:2000 in rinsing buffer with 2.2% skimmed milk powder). After five rinses, 0.07 % orthophenylene diamine and 0.22 % hydrogen peroxide in citrate buffer were added. After incubation, the reaction was stopped by the addition of 50 µl of 2.5 N HCl. The optical density was determined in a micro-ELISA reader at the a wavelength of 492 nm. The cut-off values for each ELISA were calculated as the mean OD value obtained in ELISA using the sera of 26 (LPS ELISA) or 13 (whole germ ELISA) non-infected control chicks, increased with five times the standard deviation. The experiments were performed *in duplo* and the mean value of the two measurements was calculated. Antibodies directed against *S. enteritidis* phage type 4 LPS were present in 3 out of 11 chicks tested (see Table 7). Using the complete germ as antigen, antibodies could be detected, in the serum of 9 out of 11 chicks (see Table 7). This clearly demonstrates that oral vaccination with EZ1263 induces efficient seroconversion in chicks.

Table 7 : Number of seropositive chicks at 4 weeks post inoculation

	ELISA using *S. enteritidis* LPS		ELISA using complete *S. enteritidis*	
Strain	Number of positive chicks	Total number of chicks	Number of positive chicks	Total number of chicks
EZ1263	3	11	9	11
76Sa88	1	2	2	2

Example 6 : Transfer of the attenuating mutation of EZ1263 into wild type S. enteritidis and S. typhimurium

The fact that the transposon miniTn*5lacZ2* insertion in EZ1263 was indeed the cause of the attenuation of this strain was established by generalised transduction of the transposon-induced allele into wild type *S. enteritidis* and *S. typhimurium*, using bacteriophage P22HT*int*$^-$ (Schmieger H., Phage P22 mutants with increased or decreased transduction abilities. *Mol.Gen.Genet.* 119:75-88, 1972). The P22-sensitive virulent bacteria *S. enteritidis* 76Sa88 and *S. typhimurium* 405Sa91, a clinical calf isolate obtained from the Veterinary and Agrochemical Research Centre (Groeselenberg 99, B-1180 Ukkel, Belgium), were used as recipients for the transduction.

Transducing bacteriophage stocks were prepared by incubating 10^4 plaque forming units of bacteriophage P22HT*int*$^-$ with 100 µl of an overnight culture of *S. enteritidis* EZ1263 in LB medium at 37 °C for 15 min. Subsequently, 4 ml of top agarose (8 g NaCl, 2 ml 1M $MgSO_4$ and 6 g agarose per litre) were added and the mixture was poured on top of a fresh LB plate. After overnight incubation at 37 °C, 5 ml of λ buffer (10 mM Tris-HCl

pH 7.5; 100 mM NaCl; 10 mM $MgCl_2$) were added and the plates were gently shaken at room temperature for 2-5 hours to allow the bacteriophages to diffuse. The liquid was subsequently removed with a pipette and 200 µl of chloroform were added. After incubation at 37 °C for 10 min., the suspension was centrifuged (Sorvall SS34 rotor, 15 min, 6000 rpm, 4 °C) and the resulting supernatant was stored at 4 °C in a sterile glass bottle with a few drops of chloroform. This stock was titrated by spotting 20 µl samples of serial dilutions on an LB plate, with a top layer of 100 µl of an overnight broth culture of *S. enteritidis* 76Sa88 in top agarose, and counting the number of resulting plaques.

For the transduction, 200 µl of an overnight culture of the recipient bacteria *S. enteritidis* 76Sa88 and *S. typhimurium* 405Sa91 were spun down and resuspended in 80 µl of LB medium. A 10 µl sample of an appropriate dilution of the transducing lysate, giving a multiplicity of infection of below 1, was added and the mixture was incubated for 10-15 min at 37 °C. Subsequently, 4 ml of top agarose was added and the mixture was poured on top of a freshly prepared Petri dish containing two equal layers of culture medium. The bottom LB layer contained 200 mg/l of kanamycin (to select for the presence of the kanamycin resistance gene of the miniTn*5lacZ2*) and the 12,5 ml top layer contained 20 mM EGTA (ethylene glycol-bis(β-amino-ethyl-ether) N,N,N',N'-tetra-acetic acid), a calcium chelating compound preventing further infection by P22HT*int*$^-$. After incubation at 37 °C for 24 hours, the resulting kanamycin resistant colonies were purified repeatedly by streaking on LB medium with 100 mg/l kanamycin and 10 mM EGTA. Transductants in *S. enteritidis* 76Sa88 and *S. typhimurium* 405Sa91 were readily obtained

using this technique. These data show that the attenuating mutation of strain EZ1263 can be transferred between *Salmonella* strains by standard genetic techniques.

Example 7 : Attenuated phenotype of a Salmonella typhimurium strain harbouring the attenuating mutation of S. enteritidis EZ1263

To test whether the mutation of *S. enteritidis* EZ1263 also induces attenuation in other *Salmonella* serotypes, the virulence of the transductant strain *S. typhimurium* 1263ST405, obtained by transduction of the miniTn*5lacZ2*-generated mutation of *S. enteritidis* EZ1263 into wild type *S. typhimurium* 405Sa91 (see Example 6), was tested. Oral infection of Balb/c mice with about $1.9\ 10^8$ colony forming units (cfu) of *S. typhimurium* 1263ST405 was performed as described previously (see Example 4). The morbidity and mortality data (see Table 8) indicate that the attenuated phenotype of *S. enteritidis* EZ1263 is linked to the transposon insertion. In addition, the results prove that the gene that is inactivated in *S. enteritidis* EZ1263 is also required for the virulence of *S. typhimurium*.

Table 8 : Oral infection of mice with a *S. typhimurium* strain harbouring the attenuating mutation of *S. enteritidis* EZ1263

Strain	Dose (cfu)	Number of surviving mice	Dead days after inoculation
S. typhimurium 1263ST405	$1.89\ 10^8$	3/3	/
S. typhimurium 405Sa91 (wild type)	$2.44\ 10^8$	0/3	7, 8, 10
Negative control	0	5/5	/

Example 8 : Induction of humoral immunity against both *S. enteritidis* and F17 fimbriae after oral vaccination of mice *with S. enteritidis* EZ1263 harbouring a plasmid encoding the production of F17 fimbriae

To test whether *S. enteritidis* EZ1263 can be used as a carrier for foreign antigens in the production of recombinant live vaccines, the plasmid pPLHD54 (Lintermans P., Karakterisatie van de F17 en F111 fimbriae van *Escherichia coli* en genetische analyse van de F17 genkluster, Proefschrift tot het verkrijgen van de graad van geaggregeerde van het hoger onderwijs, RUG, 1990), encoding the production of F17 fimbriae, was introduced into *S. enteritidis* EZ1263.

To avoid excessive restriction, the plasmid was first introduced by electroporation (O'Callaghan D. and Charbit A., Mol Gen Genet 223: 156-158, 1990) into a *S. typhimurium hsd* mutant (Nakayama K., Kelly S.M., Curtiss III R., Bio/Technology 6:693-697, 1988). Plasmid DNA was subsequently prepared from a transformant (JETstar 2.0 Plasmid MIDI Kit, Genomed) and used to electroporate EZ1263.

To test the immunogenicity of the resulting strain *S. enteritidis* EZ1263(pPLHD54), 5-6 weeks old female Balb/c mice were orally vaccinated with about 10^8 colony forming units per mouse as described previously (see Example 4). The vaccination was repeated after 3 weeks.

Blood samples were collected before the first immunisation and at different times after the second infection. The serum was separated by incubation of the sample for 1 hour at 37 ^{o}C followed by incubation for 2 hours at 4 ^{o}C and two centrifugations at 12.000 rpm in an

Eppendorf micro-centrifuge and stored at -20 °C.

Microtitre plates (96 wells) were coated with F17 fimbriae or *S. enteritidis* LPS (Sigma Chemie, lyophilised powder prepared by phenol extraction) using an antigen solution (2 µg/ml, 100 µl/well) in PBS for 1 hour at 37 °C. The plates were rinsed three times with PBS containing 1% Tween 80. Subsequently, 200 µl per well of a 5 mg/ml solution of bovine serum albumin (BSA) in PBS were added and the plates were incubated at 37 °C for 30 min. The plates were rinsed again three times with PBS containing 1% Tween 80.

The sera were diluted (1:100, 1:300, 1:900 after vaccination and 1:10 and 1:100 for the preimmune sera) in PBS. After addition of 50 µl of serum in each well, the plates were incubated for 1 hour at 37 °C and rinsed 6 times with PBS containing 1% Tween 80. Subsequently, 100 µl of a 1:1000 dilution of the goat anti mouse immunoglobulin conjugated with horseradish peroxidase were added. The plates were incubated for 1 hour at room temperature and washed 6 times with PBS containing 1% Tween 80. Subsequently, 100 µl substrate solution (TMB Peroxidase EIA Substrate Kit, Bio-Rad) were added in each well. The reaction was stopped after 15 min by the addition of 100 µl 1 M H_3PO_4. The optical density was determined in a micro-ELISA reader at the wavelength of 450 nm. The cut-off value for each ELISA was 2.5 times the OD value of the pre-immune serum.

The results of the ELISA test showed that antibodies directed against F17 fimbriae (Figure 3) and against *S. enteritidis* LPS (see Figure 4) were present in both of the tested mice (1 = mouse 1; 2 = mouse 2), and are clearly above the cut-off (3). The antibody titre remained high for at least 50 days. This clearly demonstrates that

oral vaccination with EZ1263 expressing F17 fimbriae induces the production of antibodies directed against both *Salmonella* LPS and F17 fimbriae. EZ1263 can therefore be used as a carrier for the expression of foreign epitopes.

Example 9 : Conversion of the transposon insertion mutation of S. enteritidis EZ1263 into a deletion

To avoid any reversion of the attenuating mutation present in EZ1263 and to remove the transposon sequence with its kanamycin resistance gene from this strain, a deletion of the relevant sequence will be introduced by homologous recombination. The 7-8 kb *Bam*HI fragment hybridising with the EZ1263-probe (see example 2) was cloned in the *Xho*I site of pACYC177 after partial fill-in of the *Bam*H1 and *Xho*I sticky ends. This resulted in the plasmid pGV4484. An internal *Pst*I deletion, removing the 1.6 kb *Pst*I fragment in which the minitransposon is inserted in strain EZ1263, was introduced in the *Bam*HI insert of this plasmid by *Pst*I digestion followed by self-ligation. This resulted in the deletion of the complete open reading frame that was interrupted by the transposon insertion.

The fragment carrying the *Pst*I deletion will be ligated into a suitable site in the suicide vector pUT (Herrero M., de Lorenzo V., Timmis K.N., J. Bacteriol. 172 (11): 6557-6567, 1990), that is unable to replicate autonomously in *Salmonella*, and transformed in strain *E. coli* S17-1(λpir). The suicide plasmid, carrying the deletion, will be mobilised to *Salmonella* strain EZ1263 or 1263SEWT (a rifampicin sensitive strain obtained by P22-mediated transduction of the transposon insertion of EZ1263 into wild type S. enteritidis 76Sa88, as described in

Example 6). The mobilisation will be performed by overnight incubation of a mixture of 100 µl of the donor and recipient strains. The integration of the suicide vector into the *Salmonella* genome, by a single recombination between homologous sequences, will be selected on LB medium containing 100 µg/l rifampicin, to counter-select the donor *E. coli* strain S17-1(λpir), and 100 µg/ml carbenicillin (marker of the suicide plasmid pUT) when EZ1263 is used as a recipient or on Minimal A medium (Miller, J.H. *Experiments in Molecular Genetics*, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1972. pp. 1-466) containing 100 µg/ml carbenicillin when 1263SEWT is the recipient. After colony purification on selective medium, several transconjugant *Salmonella* strains will be grown in liquid LB medium without antibiotics and plated on LB medium without antibiotics. The resulting colonies (about 500 per Petri dish) will be replica-plated on LB medium supplemented with carbenicillin (100 µg/ml), on LB medium supplemented with kanamycin (50 µg/ml) and on LB medium without antibiotics. Double recombinants will be identified as sensitive to the antibiotics carbenicillin and kanamycin. The presence of the deletion is subsequently confirmed by Southern DNA hybridisation using the deleted 1.6 kb *Pst*I fragment as a probe.

Example 10 : Introduction of a supplementary attenuating mutation and construction of a double deletion mutant

Combination of the *Pst*I deletion of *orf*X (see example 9) with a supplementary attenuating mutation to improve safety of the vaccine, will be carried out by transduction of a supplementary mutation, using bacteriophage P22HTint⁻, to the EZ1263-derived deletion

mutant (see example 6 for methodology of P22 transduction). This supplementary mutation can be a mutation in the *S. enteritidis spiC* gene as seen in mutant EZ870 or a mutation in the *S. enteritidis aroC* gene as seen in mutant EZ482 or in any other suitable gene.

EZ870 is a miniTn*5lacZ2* instertion mutant of *S. enteritidis* 76Sa88Rif^R and was constructed using the method described in example 1. β-galactosidase expression was induced by culture of EZ870 on LB medium with 10 % newborn calf serum (Sigma), by culture on the defined medium Minimal A (Miller, J.H. *Experiments in Molecular Genetics*, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1972. pp. 1-466), by culture on LB containing the iron chelator dipyridyl (0.2 mM) and by culture on LB containing 0.1 mM of the iron chelator diethylene triamine penta-acetate (DTPA). This induction pattern suggested that the corresponding gene might also be expressed *in vivo* after infection of the animal host, as is predicted for a virulence factor. Therefore the pathogenicity of strain EZ870 was tested orally (following the method described in example 4) and intraperitoneally (following the method described in example 3) in Balb/c mice. All of the infected animals survived without any clear disease symptoms, while all control mice infected with the wild type *S. enteritidis* 76Sa88 under identical conditions were killed by the infection (see Table 9).

The fact that the transposon miniTn*5lacZ2* insertion in EZ870 was indeed the cause of the attenuation of this strain was established by generalised transduction of the transposon-induced allele into wild type *S. enteritidis* 76Sa88 and *S. typhimurium* 405Sa91, using bacteriophage P22HTint$^-$ and following the method described in example 6.

Transductants in *S. enteritidis* 76Sa88 and *S. typhimurium* 405Sa91 were readily obtained using this technique. These data show that the attenuating mutation of strain EZ870 can be transferred between *Salmonella* strains by standard genetic techniques. Virulence of 870SEWT (transductant to *S. enteritidis* 76Sa88) and 870ST405 (transductant to *S. typhimurium* 405Sa91) was tested by oral infection of Balb/c mice following the method described in example 4. All of the infected animals survived, while all control mice infected with the wild type *S. enteritidis* 76Sa88 or *S. typhimurium* 405Sa91 under identical conditions were killed by the infection (see Table 9).

Table 9: Results of virulence tests of *S. enteritidis* EZ870 and of *S. typhimurium* and *S. enteritidis* strains harbouring the attenuating mutation of EZ870

Strain	Mode of infection	Dose (cfu)	Number of surviving mice
EZ870*	oral	$1.3\ 10^8$	3/3
76Sa88*	oral	$1.7\ 10^8$	0/3
EZ870**	oral	$4.8\ 10^8$	3/3
76Sa88**	oral	$1.4\ 10^8$	0/3
EZ870	intraperitoneal	$2.1\ 10^2$	3/3
76Sa88	intraperitoneal	$2.3\ 10^2$	0/3
870SEWT	oral	$4.4\ 10^8$	3/3
76Sa88	oral	$3.5\ 10^8$	0/3
870ST405	oral	$2.0\ 10^8$	3/3
405Sa91	oral	$2.4\ 10^8$	0/3

*, **: Results of two independent experiments

To analyse the mutated gene in EZ870, total genomic DNA of the mutant was prepared (Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Seidman J.D., Smith J.A. and Struhl K. *Current Protocols in Molecular Biology*. Wiley Interscience, 1987), digested to completion with the restriction enzyme *EcoRV*. The further steps in the IPCR procedure were performed as described in example 2. The IPCR product was reamplified with nested primers as described in example 2.

After re-amplification, the 0.3 kb PCR product was cloned in the *Sma*I site of the plasmid vector pUC18 using the SureClone™ Ligation kit (Pharmacia Biotech) according to the instructions of the manufacturer.

The 188 nucleotides upstream of the transposon were sequenced using the pUC forward and reverse primers using the SequiTherm™ cycle sequencing kit (Epicentre Technologies). A search for homologous sequences in the bacterial DNA database was done with the resulting nucleotide sequence SEQ ID NO 13, using the FastA programme (Sequence Analysis Software Package, Genetics Computer Group, Inc.) and revealed that the transposon in the mutant EZ870 is inserted in a *S. enteritidis* nucleotide sequence that is homologous (98.4 % of identical basepairs in a 188 bp overlap) to the *S. typhimurium* gene *spiC* (Accession number U51927, Ochman H., Soncini F.C. Solomon F. and Groisman E.A., Proc. Natl. Acad. Sci. U.S.A. 93, 7800-7804, 1996). This proves that the *S. enteritidis* gene that is homologous to *spiC* is necessary for full virulence of *S. enteritidis* in Balb/c mice

A deposit has been made according to the Budapest Treaty for the micro-organism *Salmonella*

enteritidis EZ870 under deposit number LMGP-18484 at the BCCM/LMG Culture Collection, Laboratorium voor Microbiologie, Ledeganckstraat 35, B-9000 Gent (Belgium)

EZ482 is a miniTn*5lacZ2* instertion mutant of *S. enteritidis* 76Sa88Rif^R and was constructed using the method described in example 1. The mutant did not grow on minimal medium A (Miller, J.H. *Experiments in Molecular Genetics*, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1972. pp. 1-466). This suggested that the mutated gene in EZ482 was involved in a biosynthetic pathway. The pathogenicity of strain EZ485 was tested by oral infection of Balb/c mice with 3.5 10^8 cfu of EZ482 (following the method described in example 4). All of the infected animals survived without any clear disease symptoms, while all control mice infected with the wild type *S. enteritidis* 76Sa88 under identical conditions were killed by the infection (see Table 10).

The three mice that were orally vaccinated with strain EZ482 were orally challenged with wild type *S. enteritidis* 76Sa88 (by the method described in example 4). The dose of administered *S. enteritidis* 76Sa88 was 2.7 10^8 colony forming units respectively. The challenge was carried out 21 days after the oral vaccination with EZ482. The vaccinated mice survived the challenge infection without showing clear disease symptoms, while all non-vaccinated control mice that were orally challenged under identical conditions were killed (see Table 10).

Table 10: Results of virulence test of *S. enteritidis* EZ482 and induction of protective immunity against *S. enteritidis* after oral vaccination of mice with *S. enteritidis* EZ482

previous infection	Strain	Mode of infection	Dose (cfu)	Number of surviving mice
none	EZ482	oral	$3.5\ 10^8$	3/3
none	76Sa88	oral	$4.4\ 10^8$	0/3
EZ482	76Sa88	oral	$2.7\ 10^8$	3/3
none	76Sa88	oral	$2.7\ 10^8$	0/3

To analyse the mutated gene in EZ482, total genomic DNA of the mutant was prepared (Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Seidman J.D., Smith J.A. and Struhl K. *Current Protocols in Molecular Biology*. Wiley Interscience, 1987), digested to completion with the restriction enzyme *Taq*I. The further steps in the IPCR procedure were performed as described in example 2. The IPCR product was reamplified with nested primers as described in example 2.

After re-amplification, the 0.7 kb PCR product was cloned in the *Sma*I site of the plasmid vector pUC18 using the SureClone™ Ligation kit (Pharmacia Biotech) according to the instructions of the manufacturer.

The region upstream of the transposon was sequenced with the pUC forward and reverse primers using the SequiTherm™ cycle sequencing kit (Epicentre Technologies). A search for homologous sequences in the bacterial DNA database was done with the resulting nucleotide sequence, using the FastA programme (Sequence

Analysis Software Package, Genetics Computer Group, Inc.) and revealed that the transposon in the mutant EZ482 is inserted in a *S. enteritidis* nucleotide sequence that is homologous (90.9 % of identical basepairs in a 88 bp overlap for the sequence obtained with the reverse pUC sequencing primer and 87.6% of identical basepairs in a 186 bp overlap for the sequence obtained with the forward pUC sequencing primer) to the *S. typhi* chorismate synthase gene *aroC* (accession number M27715).

Example 11 : Presence of the DNA sequence that is mutated in EZ1263 in other bacteria

The presence of the gene that is mutated in EZ1263 in the genome of various *Salmonella* strains and other *Enterobacteriaceae* was investigated by two different strategies: DNA hybridisation and PCR analysis with specific primers. Except where mentioned, the experiments were performed using standard procedures (Sambrook J., Fritsch E.F., Maniatis T., Molecular cloning, a laboratory manual, Second edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989). Total genomic DNA of the relevant bacterial cultures, all obtained from the collection of the Veterinary and Agrochemical Research Centre (Groeselenberg 99, B-1180 Ukkel, Belgium), was isolated as described in Example 2.

i. *Southern DNA hybridisation*

After total digestion by the restriction endonucleases *Eco*RI or *Hin*dIII, 4 µg of total genomic DNA was separated on a 0.8 % agarose gel and transferred onto a nylon membrane (Hybond-N, Amersham) as indicated by the supplier ("Blotting and hybridisation protocols for Hybond™ Membranes, Amersham).

The filter was pre-hybridised for 2 hours at 65 °C in a solution consisting of 5.8 ml H_2O, 3 ml 20×SSC (3 M NaCl, 0.3 M Na_3-Citrate), 0.5 ml 100 x Denhardt's solution (2%[w/v]BSA, 2% [w/v] Ficoll™ and 2% polyvinyl-pyrollidone), 0.5 ml 10% SDS and 0.2 ml denatured herring sperm DNA (1 mg/ml).

The probe was prepared by radio-labelling 25 ng of the IPCR fragment of the mutant EZ1263 with [α-^{32}P]dCTP using the Amersham RPN 1601Y Multiprime DNA Labelling Kit. The labelled DNA was separated from the free nucleotides using a Sephadex PD-10 G-25M column. The column was first equilibrated twice with 5 ml buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH8). The labelled DNA was eluted using the same buffer and the most radio-active fractions were pooled.

The labelled probe was denatured (5 min at 90 °C), cooled on ice for 10 min. and added to the pre-hybridised filter. The filter was incubated overnight at 65 °C. The non-hybridised probe was removed by washing as described in the Amersham protocol. The signal was detected by putting a Fuji X-ray film on top of the membrane and incubating at -70 °C, for 2-5 hours or overnight.

ii. PCR analysis

The genomic DNA was diluted 1:50 and used for a PCR amplification using the following reaction mixture: 10 µl diluted genomic DNA, 1 µl 20 µM primer 1263-1 (Table 1), 1 µl 20 µM primer 1263-2B (Table 1), 4 µl of a solution containing 2.5 mM of each dNTP, 5 µl SuperTaq buffer and 0.1 µl SuperTaq in a total volume of 50 µl. The PCR reaction consisted of 25 cycles of 94 °C for 10 sec, 55 °C for 20 sec and 72 °C for 90 sec. The PCR products were detected on a 0.8% agarose gel.

iii. Results

Using hybridisation as well as PCR, comparable results were obtained. The data, presented in Table 11, show that nucleotide sequences homologous with SEQ ID 01 are detected only in *Salmonella* strains. This indicates that the *Salmonella* mutant EZ1263 carries a mutation in a virulence gene that is specific for *Salmonella*.

Table 11 : Detection of the DNA sequence that is mutated in EZ1263 in other bacteria

Species or serotype	Strain	DNA hybridisation		PCR
		EcoRI	HindIII	
Aeromonas hydrophilla	2663	-	-	-
Aeromonas hydrophilla	2717	-	-	-
Bordetella bronchiseptica	2790S25	-	-	-
Citrobacter anomaloniticus	2688	-	-	-
Citrobacter anomaloniticus	2512	-	-	-
Enterobacter cloacae	2811S	-	-	ND
Escherichia coli O:1	Ørskov et al., 1977[1]	-	-	-
Escherichia coli O:86	Ørskov et al., 1977[1]	-	-	-
Pasteurella haemolytica	2589S	-	-	-
Plesiomonas shigelloides	2716	-	-	-
Proteus mirabilis	256ani	-	-	ND
Salmonella enteritidis	76Sa88	+	+	+
Salmonella hadar	373Sa95	+	+	+
Salmonella infantis	642Sa95	ND	ND	+
Salmonella montevideo	480Sa95	ND	ND	+
Salmonella senftenberg	402Sa95	ND	ND	+
Salmonella typhimurium	405Sa91	+	+	+

ND = not done

[1] Ørskov I., Ørskov F., Jann B., Jann K., Bacteriol. Rev. 41:667-710, 1977

CLAIMS

1. A vaccine for inducing an immune response to a *Salmonella* strain in an animal, including a human, characterised in that it comprises a pharmaceutically acceptable carrier and a genetically modified *Salmonella* strain which is in an amount effective to produce an immune response in said animal, including human, and comprises a modification in its wild type DNA sequence SEQ ID NO 09, in any of the DNA sequences from the same operon as a wild type DNA sequence selected from the group consisting of SEQ ID NO 01, SEQ ID NO 14, SEQ ID NO 15 and SEQ ID NO 16, and/or in any regulatory sequences of any of the said DNA sequences.

2. A vaccine according to claim 1, characterised in that the genetically modified *Salmonella* strain contains an isolated *Salmonella*-foreign nucleotide sequence encoding a *Salmonella*-foreign antigen and in that said genetically modified *Salmonella* strain is in an amount effective to produce an immune response to said *Salmonella*-foreign antigen in said animal, including human.

3. A vaccine according to claim 1 or 2, characterised in that the modification in the DNA sequence SEQ ID NO 09 is an insertion, a deletion or a substitution of at least one nucleotide in the wild type DNA sequence SEQ ID NO 09.

4. A vaccine according to any of the preceding claims, characterised in that the *Salmonella* strain is selected from the group consisting of: *Salmonella enteritidis*, *Salmonella typhimurium*, *Salmonella choleraesuis*, *Salmonella dublin*, *Salmonella paratyphi*, *Salmonella typhi*, *Salmonella hadar*, *Salmonella infantis*, *Salmonella montevideo* and *Salmonella senftenberg*.

5. A vaccine according to any one of the preceding claims, characterised in that the modification is

in SEQ ID NO 1.

6. A vaccine according to claim 5, characterised in that the *Salmonella* strain is the *Salmonella enteritidis* EZ1263 having the deposit number LMGP-18112.

7. A vaccine according to any one of the preceding claims, characterised in that the genetically modified *Salmonella* strain further comprises a supplementary genetic modification in the spiC, aro, pur, dap, pab, sipC, phoP, phoQ and/or pagC nucleotide sequences.

8. An isolated or synthetic virulent DNA sequence, characterised in that it has at least 55 % homology with the wild type DNA sequence SEQ ID NO 09 between positions 163 and 3580 or its complementary strand.

9. An isolated or synthetic virulent DNA sequence as in claim 8, characterised in that it has at least 70 % homology with the wild type DNA sequence SEQ ID NO 09 between positions 163 and 3580 or its complementary strand.

10. An isolated or synthetic virulent DNA sequence as in claim 8, characterised in that it has at least 85 % homology with the wild type DNA sequence SEQ ID NO 09 between positions 163 and 3580 or its complementary strand.

11. An isolated or synthetic virulent DNA sequence as in claim 8, characterised in that it is identical with the wild type DNA sequence SEQ ID NO 09 between positions 163 and 3580 or its complementary strand.

12. An isolated or synthetic virulent DNA sequence, characterised in that it has at least 40% homology with the wild type DNA sequence SEQ ID NO 01 or its complementary strand.

13. An isolated or synthetic virulent DNA

sequence as in claim 12, characterised in that it has at least 55 % homology with the wild type DNA sequence SEQ ID NO 01 or its complementary strand.

14. An isolated or synthetic virulent DNA sequence as in claim 12, characterised in that it has at least 70 % homology with the wild type DNA sequence SEQ ID NO 01 or its complementary strand.

15. An isolated or synthetic virulent DNA sequence as in claim 12, characterised in that it has at least 85 % homology with the wild type DNA sequence SEQ ID NO 01 or its complementary strand.

16. An isolated or synthetic virulent DNA sequence as in claim 12, characterised in that it is homologous with the wild type DNA sequence SEQ ID NO 01 or its complementary strand.

17. An isolated or synthetic virulent amino acid sequence, possibly encoded by the virulent DNA sequence according to any of the claims 12 to 16, and that has at least 30 % homology with the wild type amino acid sequence SEQ ID NO 02.

18. An isolated or synthetic virulent amino acid sequence such as in claim 17, characterised in that it has at least 50 % homology with the wild type amino acid sequence SEQ ID NO 02.

19. An isolated or synthetic virulent amino acid sequence such as in claim 17, characterised in that it has at least 70 % homology with the wild type amino acid sequence SEQ ID NO 02.

20. An isolated or synthetic virulent amino acid sequence such as in claim 17, characterised in that it has at least 90 % homology with the wild type amino acid sequence SEQ ID NO 02.

21. An isolated or synthetic virulent amino acid sequence such as in claim 17, characterised in that it

is identical with the wild type amino acid sequence SEQ ID NO 02.

22. Preparation method of an avirulent *Salmonella* strain, comprising the steps of :

- identifying a "virulent" nucleotide sequence in the genome of a *Salmonella* strain by any method based on the use of nucleotide sequence SEQ ID NO 09 or the complementary strand thereof, such as hybridisation or amplification by the polymerase chain reaction with a probe or primers having at least 12 nucleotides and which shows at least 10 identical nucleotides with a corresponding portion of SEQ ID NO 09 or its complementary strand or which shows more than 50% homology with a corresponding portion of SEQ ID NO 09 or its complementary strand.
- inducing a modification in said "virulent" nucleotide sequence,
- recovering an obtained avirulent *Salmonella* strain having said modification in its "virulent" sequence, and
- possibly inducing a genetic modification in another nucleotide sequence which belongs to another operon than SEQ ID NO 09, preferably in the spiC, aro, pur, dap, pab, sipC, phoP, phoQ and/or pagC gene and recovering the obtained avirulent *Salmonella* strain having said both genetic modifications.

23. The method according to claim 23, characterised in that the modification in said sequences is an insertion, a deletion and/or a substitution of at least one nucleotide in said sequences.

24. A method for inducing an immune response to a *Salmonella* strain in an animal, including a human, comprising administering a live, genetically modified *Salmonella* strain to said animal, including human,

wherein said genetically modified *Salmonella* strain is in an amount effective to produce an immune response.

25. The method according to claim 24, wherein said genetically modified *Salmonella* strain is administered in a pharmaceutically acceptable carrier.

26. Use of the vaccine according to any of the preceding claims 1 to 7 for the manufacture of a medicament for inducing an immune response to a *Salmonella* strain in an animal, including a human.

27. Use according to claim 26, characterised in that the immune response to the *Salmonella* strain in the animal, including the human, is a humoral, local and/or cellular immune response.

28. A vaccine for inducing an immune response to a *Salmonella* strain in an animal, including a human, said vaccine comprising a pharmaceutically acceptable carrier and one or more genetically modified *Salmonella* enteritidis strain(s) in an amount effective to produce said immune response (humoral, local and/or cellular immune response) and wherein said genetically modified *Salmonella* enteritidis strain comprises a modification in its wild type spiC DNA sequence, its complementary strand, or in a homologous sequence.

29. A vaccine according to claim 28, characterised in that said modification is in SEQ ID NO 13.

30. A vaccine according to claim 28 or 29, characterised in that said genetically modified *Salmonella* enteritidis is EZ870 having the deposit number LMGP-18484.

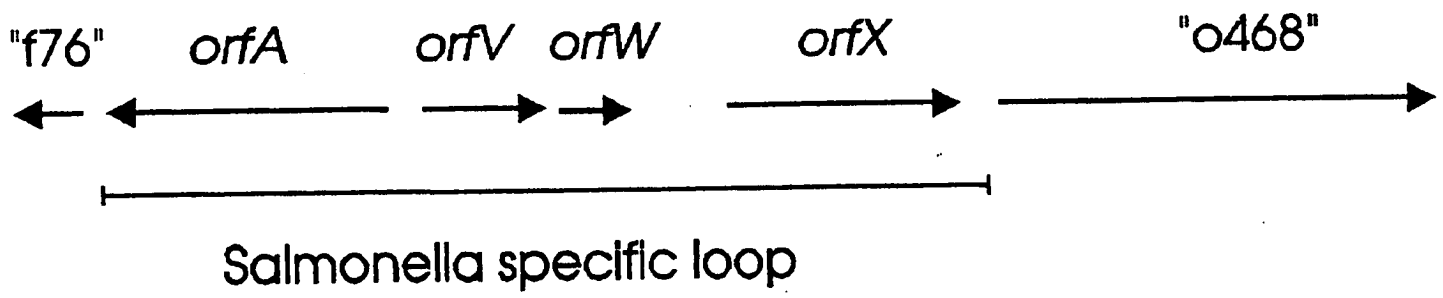
"f76"
orfA
orfV
orfW
orfX
"o468"
Salmonella specific loop

FIG. 1

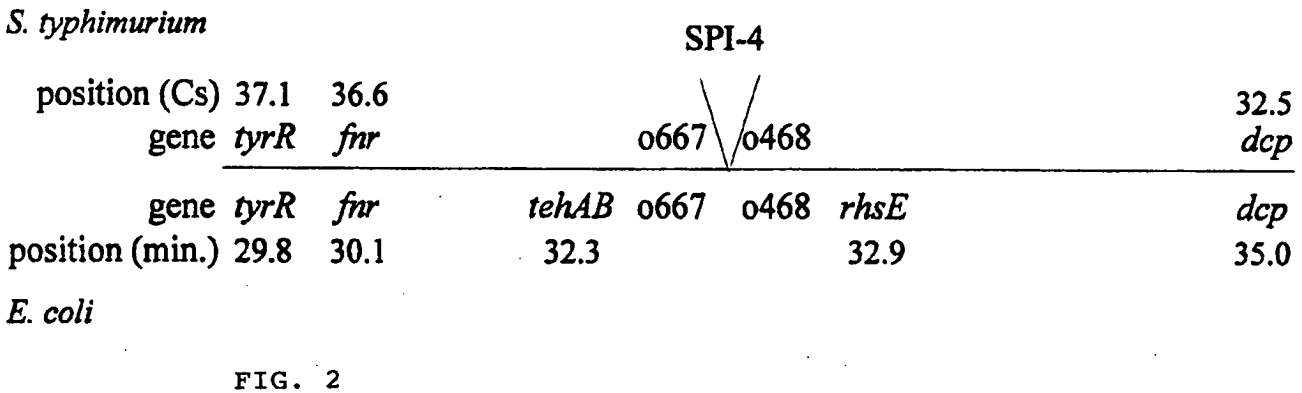
S. typhimurium
SPI-4
position (Cs) 37.1 36.6 32.5
gene tyrR fnr o667 o468 dcp
gene tyrR fnr tehAB o667 o468 rhsE dcp
position (min.) 29.8 30.1 32.3 32.9 35.0
E. coli

FIG. 2

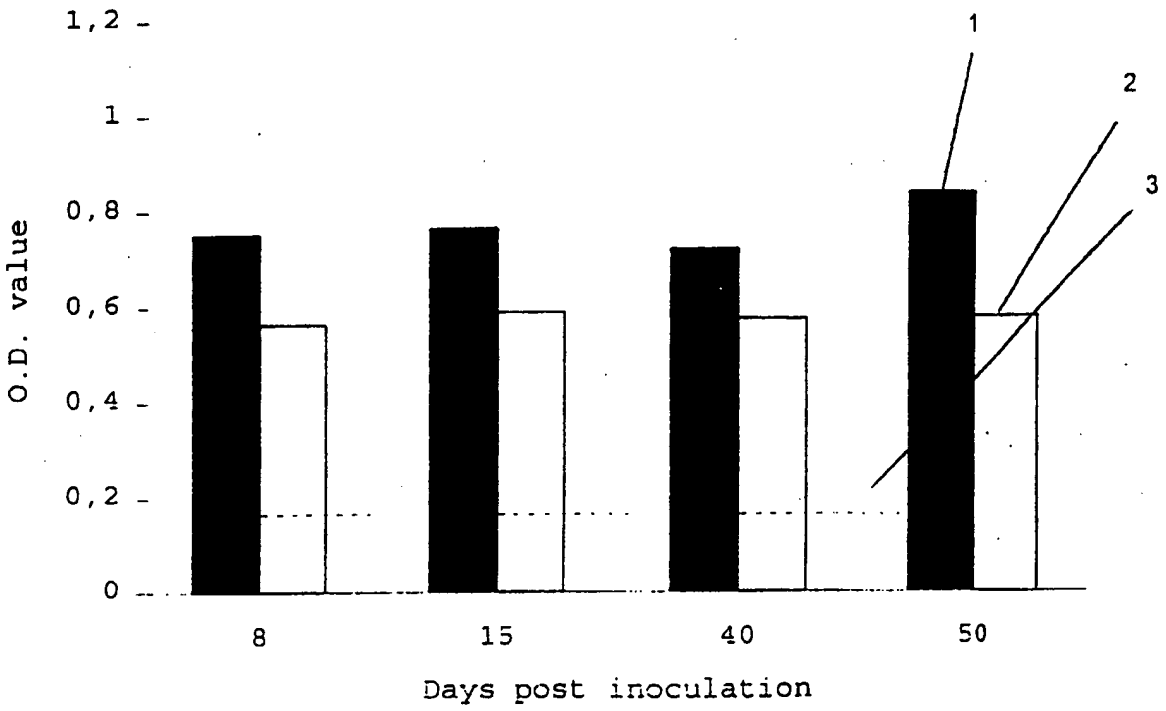

FIG. 3

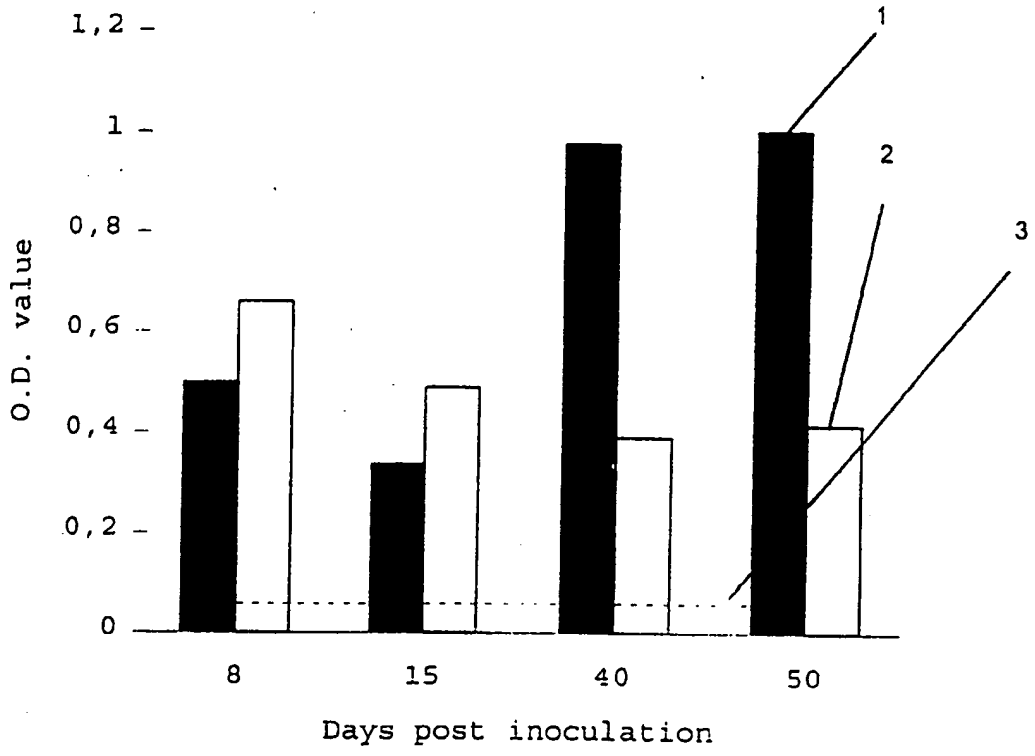

FIG. 4

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Vrije Universiteit Brussel
(B) STREET: Pleinlaan 2
(C) CITY: Brussels
(E) COUNTRY: Belgium
(F) POSTAL CODE (ZIP): B-1050

(ii) TITLE OF INVENTION: LIVE ATTENUATED SALMONELLA VACCINE

(iii) NUMBER OF SEQUENCES: 16

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 771 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: primer_bind
(B) LOCATION:380..397

(ix) FEATURE:
(A) NAME/KEY: primer_bind
(B) LOCATION:complement (172..189)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..771

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
ATG GTA AAA TGC GTA TCT TCT TTC CTC CTG TTC AGC CTG TTG TCC GTT        48
Met Val Lys Cys Val Ser Ser Phe Leu Leu Phe Ser Leu Leu Ser Val
 1               5                  10                  15

CAG GCA ATG TCA GCT GAA AAC CAT ATT GAT CTC CAC CAG CCA AAA GAC        96
Gln Ala Met Ser Ala Glu Asn His Ile Asp Leu His Gln Pro Lys Asp
            20                  25                  30

TTT GTC GAT ATT ACT ACG GTC GCC CCC GAC GTA CAG GTA GAC ATG CGA       144
Phe Val Asp Ile Thr Thr Val Ala Pro Asp Val Gln Val Asp Met Arg
        35                  40                  45
```

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TAC TTC AGT TCC CAT AAC TTT ATT GGT CGC CCC ATT AAA GGC TAT AAC        192
Tyr Phe Ser Ser His Asn Phe Ile Gly Arg Pro Ile Lys Gly Tyr Asn
     50                  55                  60

GCG CCT GTT TGC CTG TTA ACA CGA CCA GCC GCG AAC GCA GTG AAG CAG        240
Ala Pro Val Cys Leu Leu Thr Arg Pro Ala Ala Asn Ala Val Lys Gln
 65                  70                  75                  80

GTC GCC GAT CGT TTA CGC CCC TTC GGA CTT ACC TTA AAA ATA TAT GAT        288
Val Ala Asp Arg Leu Arg Pro Phe Gly Leu Thr Leu Lys Ile Tyr Asp
                 85                  90                  95

TGC TAC CGT CCG CAA AGC GCA GTG AAC GAC TTT ATC GCG TGG GCC AAA        336
Cys Tyr Arg Pro Gln Ser Ala Val Asn Asp Phe Ile Ala Trp Ala Lys
            100                 105                 110

GAT CCT TCT CAA AAC CAA ATG AAA AAC GAA TTT TAT CCG CAG GTA GAG        384
Asp Pro Ser Gln Asn Gln Met Lys Asn Glu Phe Tyr Pro Gln Val Glu
        115                 120                 125

AAA AAT CGT CTG TTT GAG GAA GGT TAT CTT GCC GCC AGA TCC GGC CAC        432
Lys Asn Arg Leu Phe Glu Glu Gly Tyr Leu Ala Ala Arg Ser Gly His
    130                 135                 140

AGT CGG GGA AGT ACG CTT GAT CTA ACG ATT GTT CCA CTT GAC AGT AAA        480
Ser Arg Gly Ser Thr Leu Asp Leu Thr Ile Val Pro Leu Asp Ser Lys
145                 150                 155                 160

ATA CCA ATA TAC GAT CCC GGA CGA CCA CTG GTG AAT TGT ACT GCG TCC        528
Ile Pro Ile Tyr Asp Pro Gly Arg Pro Leu Val Asn Cys Thr Ala Ser
                165                 170                 175

GCG GCG CAA CGC TCG CCA GAT AAT AGT CTG GAT TTT GGT ACC GGC TTT        576
Ala Ala Gln Arg Ser Pro Asp Asn Ser Leu Asp Phe Gly Thr Gly Phe
            180                 185                 190

GAC TGT TTT AGT CCG TTA TCC CAT CCC GAT AAT GTC ATG CTT ACC GCT        624
Asp Cys Phe Ser Pro Leu Ser His Pro Asp Asn Val Met Leu Thr Ala
        195                 200                 205

CAG CAA CGC GCA AAT AGG TTG TTA TTA CAA ACA TTG ATG CGT GAT GCG        672
Gln Gln Arg Ala Asn Arg Leu Leu Leu Gln Thr Leu Met Arg Asp Ala
    210                 215                 220

GGT TTT ACG CCA CTG GAT ACT GAA TGG TGG CAC TTC TCT CTT ACT CAT        720
Gly Phe Thr Pro Leu Asp Thr Glu Trp Trp His Phe Ser Leu Thr His
225                 230                 235                 240

GAA CCA TAC CCG AAC ACT TGG TTT GAC TTT CCC GTT AAG CAG AGA CCC        768
Glu Pro Tyr Pro Asn Thr Trp Phe Asp Phe Pro Val Lys Gln Arg Pro
                245                 250                 255

TGA                                                                   771
*
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 257 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Val Lys Cys Val Ser Ser Phe Leu Leu Phe Ser Leu Leu Ser Val
 1               5                  10                  15

Gln Ala Met Ser Ala Glu Asn His Ile Asp Leu His Gln Pro Lys Asp
            20                  25                  30

Phe Val Asp Ile Thr Thr Val Ala Pro Asp Val Gln Val Asp Met Arg
        35                  40                  45

Tyr Phe Ser Ser His Asn Phe Ile Gly Arg Pro Ile Lys Gly Tyr Asn
    50                  55                  60

Ala Pro Val Cys Leu Leu Thr Arg Pro Ala Ala Asn Ala Val Lys Gln
65                  70                  75                  80

Val Ala Asp Arg Leu Arg Pro Phe Gly Leu Thr Leu Lys Ile Tyr Asp
                85                  90                  95

Cys Tyr Arg Pro Gln Ser Ala Val Asn Asp Phe Ile Ala Trp Ala Lys
            100                 105                 110

Asp Pro Ser Gln Asn Gln Met Lys Asn Glu Phe Tyr Pro Gln Val Glu
        115                 120                 125

Lys Asn Arg Leu Phe Glu Glu Gly Tyr Leu Ala Ala Arg Ser Gly His
    130                 135                 140

Ser Arg Gly Ser Thr Leu Asp Leu Thr Ile Val Pro Leu Asp Ser Lys
145                 150                 155                 160

Ile Pro Ile Tyr Asp Pro Gly Arg Pro Leu Val Asn Cys Thr Ala Ser
                165                 170                 175

Ala Ala Gln Arg Ser Pro Asp Asn Ser Leu Asp Phe Gly Thr Gly Phe
            180                 185                 190

Asp Cys Phe Ser Pro Leu Ser His Pro Asp Asn Val Met Leu Thr Ala
        195                 200                 205

Gln Gln Arg Ala Asn Arg Leu Leu Leu Gln Thr Leu Met Arg Asp Ala
    210                 215                 220

Gly Phe Thr Pro Leu Asp Thr Glu Trp Trp His Phe Ser Leu Thr His
225                 230                 235                 240

Glu Pro Tyr Pro Asn Thr Trp Phe Asp Phe Pro Val Lys Gln Arg Pro
                245                 250                 255
```

*

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGGAATTCAA AGCGCCATTC GCCATTCAG 29

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GGAAGCTTTA TGGCAGGGTG AAACGCAGG 29

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CGTCTAGACG TTTTCCCAGT CACGAC 26

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GCGGATCCTT TCGGCGGTGA AATTATC 27

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ACAGACGATT TTTCTCTA 18

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CGCCCCATTA AAGGCTAT 18

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3958 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Salmonella

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:complement (352..1302)
(D) OTHER INFORMATION:/product= "orfA"

(ix) FEATURE:
(A) NAME/KEY: CDS

(B) LOCATION:1839..2237
(D) OTHER INFORMATION:/product= "orfV"

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:2270..2500
(D) OTHER INFORMATION:/product= "orfW"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GGTCTTGCTG TTTCGCCAGT AACTGGCCTG AAAACAGCGC ACAGGTCAGT AAAATCACTG 60

ATAACGTTTT TGTAAGCATC ATGCCACCTT CCAGTTACGT CGTGAGGCTA TGATGCCAAT 120

AATTTCTTTG AGTTATTATT CCACACTTCA AATTAGCGAA AACGGTTTGT CAATAAGTGC 180

GATAAGTTGT ATGAGAAAAT ATACTTATAC TCAGTATATC AATTATAAAA ATAACGAGCC 240

AATTCGTTCC ATAGTAAATC CATTATTCCC TGGGAGTATA TATTTAAAGT TATTGTATTA 300

GATGGTTTTG GTATTGCAGG GGATTGTAAA TCCATACTAT TTATGGTGTG ATCAACTCTG 360

GTGATGAGCC TCATTTTTTG TTGTTTCAAC GAGTGAAGAT AAATCTTCAG AAAAAAGGCT 420

GTTATGAACT TGTAGCGCTA CATATTCAGT AAGCTCGTTT AAACATAGCA GGCCATGAAA 480

GGAACTTTCT TCCAGCATAT TCATTAGTAT CTCATTCTTA AACAAACATG AATAAACAAT 540

TTGAGTAACT GAGCTTTGTG CATAATTTGG TTCAAACGCT GCATTCTTTA AGTTAGTATT 600

CTCAATAACC TTTGCGGCTA TTTTGTTTTT GATAGCGTCG TCAATAGGTG GTACATCTGC 660

CTGGGCAATG CCCAGATTAA TCCTGGAGGA GATGGCGGCT TTTCTTTCCT GTTCCTTCCA 720

GTCACATAAA TTACCCTTTT CTGCCATGGC GATAAGCGTC TCTTTTATAT TTTTTAGGTC 780

AACGGGAAAT GTTATACCGC CGGATAGCGA GAGTAGTAAC CGCTCAGGGA ACAAGCAACA 840

TAGCTCTTCA CAAACATAAG TGCTAGCTTT TATAGTAATA TCAGAATATC TTTTCAGGTG 900

GGCAGGCTGA GTATCAACAC AGAGCACTGT TCTATTCATT ATATCATAAT AGTAGTGATC 960

CTGACGTATC ACAATGCATA ACGGAATATC TTCGCCATCT TTAATAAAGA GATTTGTATA 1020

TTGACACTCA GCATCTTTAT AGTGCTGAAA ATGGAAATTT CCTCTGCATG ACGGAGATGA 1080

TAATTCATAT AACGCGCAAA ATAAATCAAA TAAACGTTGC GCGTCAGGCG GAGAGGCAAC 1140

ATCACATAAT TCCTTTATAT ATTGATCGGC AGTGGATCTT TGCGTGTCAC AAAAAAAATC 1200

TTTAATCCTT TCCCATAGTA ACGTAAAGAA GGAGCGTTGA GAGATAGCAC TTTGAGAAAA 1260

CATCTCACTC TTTAAAAATC CTCTCCCGAT AGTAATTGGC ATAATGTAGA CCACAAGTGA 1320

TTATATGATA CTTCATTACT GGAATAGGTG GTATTCGAAA TATTATCCCA TGTTGCCCAT 1380

CGGTTTGCCT ATCGGTGAAA CACCTGATTT TTGCTTTGTC CTGAACCGTC AACATTATTG 1440

TTCAATTGTT CAAATCGACC CGTAGCTTTA GTCATGCCCA CGCCTCCTGG CCATGAAATA 1500

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TGTCAGATAA AACGAATGAA AGTAAAACGG TTTTCTTAAT TCTCACATCA TCATGTACTA    1560

TGAGTAATGA TTAATTACGC ACTATATTAT TTTTAGAGAA AGTAAATAGT TGCTCAACCG    1620

TGTAGAAATT GTCTTATAAG AAGTTAAACT AAAAGTATTA TTAGGCTAAC AACAATGAGA    1680

TGTTTAGCGG TAGGGCAGAA GGCCAATACT GATAGTGCTT ATGATATAAA TACCTTACTC    1740

TTTAGTTTTT GTCTTAATTA TATTTGTTGT AACGATTAGC TGACGGCTTT ATTTCCAGTT    1800

GGGCGATAAA ATTATAAAAA CCTGCGAGGA GGCTCAAA ATG AAG AAA TCA GAT         1853
                                          Met Lys Lys Ser Asp
                                                260

GGT GAA ATT CAC GAA AAG ACA GCA TCC TGG GGC ATT TTG CAG TCA GAA       1901
Gly Glu Ile His Glu Lys Thr Ala Ser Trp Gly Ile Leu Gln Ser Glu
        265                 270                 275

TGG CTA AGA AAA TGT GGA CGG CTA TTA TTG CTG TTA CTT TAC CGT TTC      1949
Trp Leu Arg Lys Cys Gly Arg Leu Leu Leu Leu Leu Leu Tyr Arg Phe
    280                 285                 290

GTT ATC GGA TGG GCT TTT TTT CAA TTA CTT GCC ATG ATC GTG GCA GGG      1997
Val Ile Gly Trp Ala Phe Phe Gln Leu Leu Ala Met Ile Val Ala Gly
295                 300                 305                 310

ATA TTT TTG TTA GGC GTC TTA TTA TTT CAT CCC ATA ATA TTT GTA CAG      2045
Ile Phe Leu Leu Gly Val Leu Leu Phe His Pro Ile Ile Phe Val Gln
                315                 320                 325

ACT ATC GCA ATC ACT GAG AAG TTA AAT CAT GCT TCG CTT GAT TTA TGG      2093
Thr Ile Ala Ile Thr Glu Lys Leu Asn His Ala Ser Leu Asp Leu Trp
            330                 335                 340

CAT ATC CTT AAG TTA TGC CTA TGG CAT TAC GGT ATC ATC GCA GGG TTT      2141
His Ile Leu Lys Leu Cys Leu Trp His Tyr Gly Ile Ile Ala Gly Phe
        345                 350                 355

ATT TTT ATG GCG GAG TGT ACG TTA AGT AAA AGT ATA CGG CAG GTT CAG      2189
Ile Phe Met Ala Glu Cys Thr Leu Ser Lys Ser Ile Arg Gln Val Gln
    360                 365                 370

CGC TTG TCC AAA AAA TTT GGG GCA CAG GAT TTT TCT TCA CGC CCG TGA      2237
Arg Leu Ser Lys Lys Phe Gly Ala Gln Asp Phe Ser Ser Arg Pro  *
375                 380                 385                 390

TCCGCCGCTG CAACCTGCGG TCTTACTGAA TA ATG GCT ATG ACC AGC AGA CCG      2290
                                    Met Ala Met Thr Ser Arg Pro
                                      1               5

AAT TAT CTC GGT TCG AGG GGG ATT CTA TGT GTT TGC ACA ACT GCA GTG      2338
Asn Tyr Leu Gly Ser Arg Gly Ile Leu Cys Val Cys Thr Thr Ala Val
         10                  15                  20

AAT CGT AAT TTC AGT GCA TTA TCG CCG ACA ATC GAC GTG TTC CTC ACT      2386
Asn Arg Asn Phe Ser Ala Leu Ser Pro Thr Ile Asp Val Phe Leu Thr
     25                  30                  35

AAT TGC CTT CCT GAC TAT ATA GTG GTC TTA TCT TTG GCG AAA CAA TGT      2434
Asn Cys Leu Pro Asp Tyr Ile Val Val Leu Ser Leu Ala Lys Gln Cys
 40                  45                  50                  55
```

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TAT TTA GTT ATG GAA GGC GAC AAC AAC TGT ACT ACT GAT TAT CAA ATG      2482
Tyr Leu Val Met Glu Gly Asp Asn Asn Cys Thr Thr Asp Tyr Gln Met
                60                  65                  70

ACC TTT CTG GTC AGG TAG ACTCGCCTTT GTGAAAACAC ATCGTTATCT            2530
Thr Phe Leu Val Arg  *
            75

GTGCTCCTGA GACTCACTCC CTTGCCGCCT TTACGCAACT CGAATTATTT TGGGTATAGA    2590

ACAGGAGGCG CAGTGGTCGT ATAAGCAAAA TATAAACTCT CCGTTTGTGA TAAGGCACAG    2650

ATTACAGGGG GAATGATGTT TATTTTAACC ATCTGTTTAA GCGGTGTCCG CTAATCTTTA    2710

CCTGCTCAAA TACATAATCA CCCCTGTGAC TCTCGCGAGG TGTAACATAT GGTAAAATGC    2770

GTATCTTCTT TCCTCCTGTT CAGCCTGTTG TCCGTTCAGG CAATGTCAGC TGAAAACCAT    2830

ATTGATCTCC ACCAGCCAAA AGACTTTGTC GATATTACTA CGGTCGCCCC CGACGTACAG    2890

GTAGACATGC GATACTTCAG TTCCCATAAC TTTATTGGTC GCCCCATTAA AGGCTATAAC    2950

GCGCCTGTTT GCCTGTTAAC ACGACCAGCC GCGAACGCAG TGAAGCAGGT CGCCGATCGT    3010

TTACGCCCCT TCGGACTTAC CTTAAAAATA TATGATTGCT ACCGTCCGCA AAGCGCAGTG    3070

AACGACTTTA TCGCGTGGGC CAAAGATCCT TCTCAAAACC AAATGAAAAA CGAATTTTAT    3130

CCGCAGGTAG AGAAAAATCG TCTGTTTGAG GAAGGTTATC TTGCCGCCAG ATCCGGCCAC    3190

AGTCGGGGAA GTACGCTTGA TCTAACGATT GTTCCACTTG ACAGTAAAAT ACCAATATAC    3250

GATCCCGGAC GACCACTGGT GAATTGTACT GCGTCCGCGG CGCAACGCTC GCCAGATAAT    3310

AGTCTGGATT TTGGTACCGG CTTTGACTGT TTTAGTCCGT TATCCCATCC CGATAATGTC    3370

ATGCTTACCG CTCAGCAACG CGCAAATAGG TTGTTATTAC AAACATTGAT GCGTGATGCG    3430

GGTTTTACGC CACTGGATAC TGAATGGTGG CACTTCTCTC TTACTCATGA ACCATACCCG    3490

AACACTTGGT TTGACTTTCC CGTTAAGCAG AGACCCTGAA ACGGCGTTTT GTTGCGAAAT    3550

CTAATCATTA CGCCTGTTGG AAAGCACCAT AACAGTTAGC AAATCATTGT TAATTTTAAC    3610

AACTGATATA CACTGCGGTT GCCACCTGCA AGCAGCGCTC AATGAGATCG AATAATGAAA    3670

AAATATCAAC GTCTGGCGGA GCAAATTAGA GAACAAATCG CCTCTGGCGT TTGGCAACCC    3730

GGCGATCGAT TACCCTCGCT GAGGGAGCAG GTCGCCAGTA GCGGCATGAG TTTTATGACT    3790

GTCGGTCATG CGTACCAGTT GCTGGAAAGT CAGGGACGGA TTATCGCCCG TCCGCAATCT    3850

GGTTATTATG TCGCGCCGCA TCCGGTTTGT CGGTCAGTCG CGACGGCAGC GCACGTTATT    3910

CGGGATGAAG CCGTAGATAT CAATACCTAT ATTTTTGAGA TGCTGCAG                 3958
```

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 317 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

```
Met Pro Ile Thr Ile Gly Arg Gly Phe Leu Lys Ser Glu Met Phe Ser
 1               5                  10                  15

Gln Ser Ala Ile Ser Gln Arg Ser Phe Phe Thr Leu Leu Trp Glu Arg
            20                  25                  30

Ile Lys Asp Phe Phe Cys Asp Thr Gln Arg Ser Thr Ala Asp Gln Tyr
        35                  40                  45

Ile Lys Glu Leu Cys Asp Val Ala Ser Pro Pro Asp Ala Gln Arg Leu
    50                  55                  60

Phe Asp Leu Phe Cys Ala Leu Tyr Glu Leu Ser Ser Pro Ser Cys Arg
65                  70                  75                  80

Gly Asn Phe His Phe Gln His Tyr Lys Asp Ala Glu Cys Gln Tyr Thr
                85                  90                  95

Asn Leu Phe Ile Lys Asp Gly Glu Asp Ile Pro Leu Cys Ile Val Ile
            100                 105                 110

Arg Gln Asp His Tyr Tyr Tyr Asp Ile Met Asn Arg Thr Val Leu Cys
        115                 120                 125

Val Asp Thr Gln Pro Ala His Leu Lys Arg Tyr Ser Asp Ile Thr Ile
    130                 135                 140

Lys Ala Ser Thr Tyr Val Cys Glu Glu Leu Cys Cys Leu Phe Pro Glu
145                 150                 155                 160

Arg Leu Leu Leu Ser Leu Ser Gly Gly Ile Thr Phe Pro Val Asp Leu
                165                 170                 175

Lys Asn Ile Lys Glu Thr Leu Ile Ala Met Ala Glu Lys Gly Asn Leu
            180                 185                 190

Cys Asp Trp Lys Glu Gln Glu Arg Lys Ala Ala Ile Ser Ser Arg Ile
        195                 200                 205

Asn Leu Gly Ile Ala Gln Ala Asp Val Pro Pro Ile Asp Asp Ala Ile
    210                 215                 220

Lys Asn Lys Ile Ala Ala Lys Val Ile Glu Asn Thr Asn Leu Lys Asn
225                 230                 235                 240

Ala Ala Phe Glu Pro Asn Tyr Ala Gln Ser Ser Val Thr Gln Ile Val
                245                 250                 255

Tyr Ser Cys Leu Phe Lys Asn Glu Ile Leu Met Asn Met Leu Glu Glu
            260                 265                 270

Ser Ser Phe His Gly Leu Leu Cys Leu Asn Glu Leu Thr Glu Tyr Val
        275                 280                 285
```

```
Ala Leu Gln Val His Asn Ser Leu Phe Ser Glu Asp Leu Ser Ser Leu
    290                 295                 300

Val Glu Thr Thr Lys Asn Glu Ala His His Gln Ser  *
305                 310                 315
```

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 133 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

```
Met Lys Lys Ser Asp Gly Glu Ile His Glu Lys Thr Ala Ser Trp Gly
  1               5                  10                  15

Ile Leu Gln Ser Glu Trp Leu Arg Lys Cys Gly Arg Leu Leu Leu Leu
             20                  25                  30

Leu Leu Tyr Arg Phe Val Ile Gly Trp Ala Phe Phe Gln Leu Leu Ala
         35                  40                  45

Met Ile Val Ala Gly Ile Phe Leu Leu Gly Val Leu Leu Phe His Pro
     50                  55                  60

Ile Ile Phe Val Gln Thr Ile Ala Ile Thr Glu Lys Leu Asn His Ala
 65                  70                  75                  80

Ser Leu Asp Leu Trp His Ile Leu Lys Leu Cys Leu Trp His Tyr Gly
                 85                  90                  95

Ile Ile Ala Gly Phe Ile Phe Met Ala Glu Cys Thr Leu Ser Lys Ser
            100                 105                 110

Ile Arg Gln Val Gln Arg Leu Ser Lys Lys Phe Gly Ala Gln Asp Phe
        115                 120                 125

Ser Ser Arg Pro  *
    130
```

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 77 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

```
Met Ala Met Thr Ser Arg Pro Asn Tyr Leu Gly Ser Arg Gly Ile Leu
  1               5                  10                  15

Cys Val Cys Thr Thr Ala Val Asn Arg Asn Phe Ser Ala Leu Ser Pro
             20                  25                  30
```

Thr Ile Asp Val Phe Leu Thr Asn Cys Leu Pro Asp Tyr Ile Val Val
35 40 45

Leu Ser Leu Ala Lys Gln Cys Tyr Leu Val Met Glu Gly Asp Asn Asn
50 55 60

Cys Thr Thr Asp Tyr Gln Met Thr Phe Leu Val Arg *
65 70 75

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 195 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GAGACAGGCA TAACCATGGV CGTCCGGGTG TGCTGCAAGC AGTAGTGTCA CATAGGCAAG 60

ACAAGGCTTA GGTAAGCTTT CCAGGTCATT TAAGAACAAA GAAATAGAAA ATGCTTCTGA 120

GAAAATTTCT CCTCTGGCAG GATGCCCATC AATAGTCATT ATCCAGGATC GGCTATTACC 180

TTCGGCCTTG ATATC 195

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 951 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Salmonella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TCAACTCTGG TGATGAGCCT CATTTTTTGT TGTTTCAACG AGTGAAGATA AATCTTCAGA 60

AAAAAGGCTG TTATGAACTT GTAGCGCTAC ATATTCAGTA AGCTCGTTTA AACATAGCAG 120

GCCATGAAAG GAACTTTCTT CCAGCATATT CATTAGTATC TCATTCTTAA ACAAACATGA 180

ATAAACAATT TGAGTAACTG AGCTTTGTGC ATAATTTGGT TCAAACGCTG CATTCTTTAA 240

GTTAGTATTC TCAATAACCT TTGCGGCTAT TTTGTTTTTG ATAGCGTCGT CAATAGGTGG 300

TACATCTGCC TGGGCAATGC CCAGATTAAT CCTGGAGGAG ATGGCGGCTT TTCTTTCCTG 360

TTCCTTCCAG TCACATAAAT TACCCTTTTC TGCCATGGCG ATAAGCGTCT CTTTTATATT 420

TTTTAGGTCA ACGGGAAATG TTATACCGCC GGATAGCGAG AGTAGTAACC GCTCAGGGAA 480

CAAGCAACAT AGCTCTTCAC AAACATAAGT GCTAGCTTTT ATAGTAATAT CAGAATATCT 540

TTTCAGGTGG GCAGGCTGAG TATCAACACA GAGCACTGTT CTATTCATTA TATCATAATA 600

GTAGTGATCC TGACGTATCA CAATGCATAA CGGAATATCT TCGCCATCTT TAATAAAGAG 660

ATTTGTATAT TGACACTCAG CATCTTTATA GTGCTGAAAA TGGAAATTTC CTCTGCATGA 720

CGGAGATGAT AATTCATATA ACGCGCAAAA TAAATCAAAT AAACGTTGCG CGTCAGGCGG 780

AGAGGCAACA TCACATAATT CCTTTATATA TTGATCGGCA GTGGATCTTT GCGTGTCACA 840

AAAAAAATCT TTAATCCTTT CCCATAGTAA CGTAAAGAAG GAGCGTTGAG AGATAGCACT 900

TTGAGAAAAC ATCTCACTCT TTAAAAATCC TCTCCCGATA GTAATTGGCA T 951

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 399 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Salmonella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

ATGAAGAAAT CAGATGGTGA AATTCACGAA AAGACAGCAT CCTGGGGCAT TTTGCAGTCA 60

GAATGGCTAA GAAAATGTGG ACGGCTATTA TTGCTGTTAC TTTACCGTTT CGTTATCGGA 120

TGGGCTTTTT TTCAATTACT TGCCATGATC GTGGCAGGGA TATTTTTGTT AGGCGTCTTA 180

TTATTTCATC CCATAATATT TGTACAGACT ATCGCAATCA CTGAGAAGTT AAATCATGCT 240

TCGCTTGATT TATGGCATAT CCTTAAGTTA TGCCTATGGC ATTACGGTAT CATCGCAGGG 300

TTTATTTTTA TGGCGGAGTG TACGTTAAGT AAAAGTATAC GGCAGGTTCA GCGCTTGTCC 360

AAAAAATTTG GGGCACAGGA TTTTTCTTCA CGCCCGTGA 399

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 231 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Salmonella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

ATGGCTATGA CCAGCAGACC GAATTATCTC GGTTCGAGGG GGATTCTATG TGTTTGCACA 60

ACTGCAGTGA ATCGTAATTT CAGTGCATTA TCGCCGACAA TCGACGTGTT CCTCACTAAT 120

TGCCTTCCTG ACTATATAGT GGTCTTATCT TTGGCGAAAC AATGTTATTT AGTTATGGAA 180

GGCGACAACA ACTGTACTAC TGATTATCAA ATGACCTTTC TGGTCAGGTA G 231

BCCM™/LMG
CULTURE COLLECTION
LABORATORIUM VOOR
MICROBIOLOGIE
UNIVERSITEIT GENT
K.L.LEDEGANCKSTRAAT 35
B-9000 GENT - BELGIUM
TEL: +32-9-264.51.08
FAX: +32-9-264.53.46
BCCM.LMG@RUG.AC.BE

Office Van Malderen
Place Reine Fabiola 6/1
1080 Brussels

GENT, September 26, 1997

YOUR REF.:
OUR REF.:

Re.: deposit bacterial culture for patent purposes (Budapest Treaty)

Dear Sir,

At the request of Prof. Dr. J.-P. Hernalsteens (Eenheid Genetische Virologie, VUB), I forward you a **copy** the following documents regarding the patent deposit (Budapest Treaty) of the bacterial culture

Salmonella enteritidis **EZ1263 = LMG P-18112**

- Attestation of receipt: form BCCM/LMG/**BP/4**/97-45
- Statement of viability: form BCCM/LMG/**BP/9**/97-45

Sincerely yours,

Dr. D. Janssens
Curator BCCM/LMG

cc: Prof. Dr. J.-P. Hernalsteens, VUB

Investigations are performed to the best of our knowledge under the experimental conditions indicated. Results can not be used in judicial conflicts without written agreement of the Director.

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM LMG-COLLECTION
Page 1 of Form BCCM/LMG/BP/4/ 97-45 Receipt in the case of an original deposit

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Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure

Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the International Depositary Authority BCCM/LMG identified at the bottom of next page

***International* Form BCCM/LMG/BP/4/ 97-45**

To: Name of the depositor : Prof. J.-P. Hernalsteens

Address : Eenheid Genetische Virologie
Vrije Universiteit Brussel
Paardenstraat 65
1640 Sint-Genesius-Rode
Belgium

I. Identification of the microorganism:

I.1 Identification reference given by the **depositor:**

EZ1263

I.2 Accession number given by the **International Depositary Authority:**

LMG P-18112

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION
Page 2 of Form BCCM/LMG/BP/4/...97-45... Receipt in the case of an original deposit

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II. Scientific description and/or proposed taxonomic designation

The microorganism identified under I above was accompanied by :

(mark with a cross the applicable box(es)):

[X] a scientific description

[X] a proposed taxonomic designation

III. Receipt and acceptance

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

September 8, 1997

IV. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Dr. D. Janssens, Curator IDA

Date : September 25, 1997

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION
Page 1 of Form BCCM/LMG/BP/9/..97-45..... Viability statement

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Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure

Viability statement issued pursuant to Rule 10.2 by the International Depositary Authority BCCM/LMG identified on the following page

International **Form BCCM/LMG/BP/9/..97-45.....**

To: Party to whom the viability statement is issued:

Name : = Prof. J.-P. Hernalsteens

Address : Eenheid Genetische Virologie
Vrije Universiteit Brussel
Paardenstraat 65
1640 Sint-Genesius-Rode
Belgium

I. Depositor:

I.1 Name : Prof. J.-P. Hernalsteens

I.2 Address : Eenheid Genetische Virologie
Vrije Universiteit Brussel
Paardenstraat 65
1640 Sint-Genesius-Rode
Belgium

II. Identification of the microorganism

II.1 Accession number given by the International Depositary Authority:

LMG P-18112

II.2 Date of the original deposit (or where a new deposit or a transfer has been made, the most recent relevant date) :

September 8, 1997

III. Viability statement.

The viability of the microorganism identified under II above was tested on

September 9, 1997

(Give date. In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test).

On that date, the said microorganism was: (mark the applicable box with a cross)

☒ viable

☐ no longer viable

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION
Page 2 of Form BCCM/LMG/BP/9/97-45 Viability statement

IV. Conditions under which the viability test has been performed:

(Fill in if the information has been requested and if the results of the test were negative).

V. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) :

Dr. D. Janssens, Curator IDA

Date : September 25, 1997

PCT/BE99/00007

Office Van Malderen
Koningin Fabiola Plein 6/1
B-1083 Brussel

REÇU
14.-9-1998
OFFICE VAN MALDEREN

GENT, September 10, 1998

BCCM™/LMG
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B-9000 GENT - BELGIUM
TEL: +32-9-264.51.08
FAX: +32-9-264.53.46
BCCM.LMG@RUG.AC.BE

YOUR REF.:
OUR REF.:

Re.: deposit bacterial culture for patent purposes (Budapest Treaty)

Dear Sir, Madam,

At the request of Prof. J.-P. Hernalsteens (VUB - Eenheid Genetische Virologie - St-Genesius-Rode), I forward you a **copy** the following documents regarding the patent deposit (Budapest Treaty) of the bacterial culture

Salmonella enteritidis **EZ870 = LMG P-18484**

- ➔ Attestation of receipt: form BCCM/LMG/**BP/4/98-53**
- ➔ Statement of viability: form BCCM/LMG/**BP/9/98-53**

Sincerely yours,

Dr. D. Janssens
Curator BCCM™/LMG

cc: Prof. J.-P. Hernalsteens, VUB

Investigations are performed to the best of our knowledge under the experimental conditions indicated. Results can not be used in judicial conflicts without written agreement of the Director.

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMG-COLLECTION
Page 1 of Form BCCM/LMG/BP/4/98-53 Receipt in the case of an original deposit

---

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure

Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the International Depositary Authority BCCM/LMG identified at the bottom of next page

International **Form BCCM/LMG/BP/4/98-53**

To: **Name of the depositor** : Prof. J.-P. Hernalsteens

Address : Eenheid genetische virologie
Vrije Universiteit Brussel
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. **Identification of the microorganism:**

I.1 **Identification reference given by the depositor:**

EZ870

I.2 **Accession number given by the International Depositary Authority:**

LMG P-18484

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM LMG-COLLECTION
Page 2 of Form BCCM/LMG/BP/4/...98-53... Receipt in the case of an original deposit

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II. Scientific description and/or proposed taxonomic designation

The microorganism identified under I above was accompanied by :

(mark with a cross the applicable box(es)):

☒ a scientific description

☒ a proposed taxonomic designation

III. Receipt and acceptance

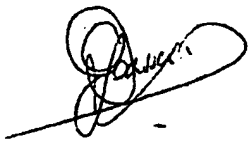
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) :

August 7, 1998

IV. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):



Dr. D. Janssens, Curator IDA

Date : September 8, 1998

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM LMG-COLLECTION
Page 1 of Form BCCM/LMG/BP/9/98-53 Viability statement

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure

Viability statement issued pursuant to Rule 10.2 by the International Depositary Authority BCCM/LMG identified on the following page

International **Form BCCM/LMG/BP/9/98-53**

To: Party to whom the viability statement is issued:

Name : Prof. J.-P. Hernalsteens

Address : Eenheid Genetische Virologie
Vrije Universiteit Brussel
Paardenstraat 65
B-1640 Sint-Genesius-Rode

I. Depositor:

I.1 Name : Prof. J.-P. Hernalsteens

I.2 Address : Eenheid Genetische Virologie
Vrije Universiteit Brussel
Paardenstraat 65
B-1640 Sint-Genesius-Rode

II. Identification of the microorganism

II.1 Accession number given by the International Depositary Authority:

LMG P-18484

II.2 Date of the original deposit (or where a new deposit or a transfer has been made, the most recent relevant date) :

August 7, 1998

III. Viability statement.

The viability of the microorganism identified under II above was tested on

: August 10-11, 1998

(Give date. In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test).

On that date, the said microorganism was: (mark the applicable box with a cross)

☒ viable

☐ no longer viable

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM LMG-COLLECTION
Page 2 of Form BCCM/LMG/BP/9/..98-53.... Viability statement

IV. Conditions under which the viability test has been performed:

(Fill in if the information has been requested and if the results of the test were negative).

V. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Microbiologie - Bacteriënverzameling (LMG)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) :

Dr. D. Janssens, Curator IDA

Date : September 8, 1998

Statement according to Rule 28 (3) EPC

The applicant informs the European Patent Office that, until the publication of the mention of grant of the European Patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, the availability referred to in paragraph 3 of Rule 28 shall be effected only by the issue of a sample to an expert nominated by the requester.

Eric VAN MALDEREN
(Authorized representative)

Declaration according to Rule 28(1)d

Prof. J.-P. Hernalsteens
Eenheid Genetische Virologie
Vrije Universiteit Brussel
Paardenstraat 65
1640 Sint-Genesius-Rode
Belgium

has made according to the Budapest Treaty a deposit of the micro-organism *Salmonella enteriditis* EZ870 under the deposit number LMG P-18484 at the BCCM/LMG Culture Collection, Laboratorium voor Microbiologie, Universiteit Gent, K. L. Ledeganckstraat 35, B-9000 Gent (Belgium).

The depositor declares that he authorises VRIJE UNIVERSITEIT BRUSSEL, Pleinlaan 2, B-1050 Brussels (Belgium) to refer to the deposited biological materials in any patent application and gives his unreserved and irrevocable consent to the deposit materials being made available to the public in accordance with the Rule 28.

Prof. J.-P. Hernalsteens

Signature : 

Date : February 3, 1999